Universidade de Aveiro, Departamento de Química
2017

Michael Santos Silva

Estudo de citometria de fluxo da regulação da população de células pela fosforilação de c-FLIP

Flow cytometric study of c-FLIP$_L$-mediated regulation of cell cycle and cell population size

Universidade de Aveiro, Departamento de Química
2017

Michael Santos Silva

Estudo de citometria de fluxo da regulação da população de células pela fosforilação de c-FLIP

Flow cytometric study of c-FLIP$_L$-mediated regulation of cell cycle and cell population size

Dissertation presented to Universidade de Aveiro and Åbo Akademi University to obtain the degree of MSc in Biochemistry, Clinical Biochemistry field, made under the supervision of Doctor John Eriksson Professor of Cell Biology, Director of Centre for Biotechnology, Chair of Turku Bioimaging, Faculty of Science and Engineering of Åbo Akademi University and Doctor Brian Goodfellow, Auxiliary Professor of the Chemistry Department of Universidade de Aveiro.

To my family, for the everlasting support

Juri

President	Cecilia Sahlgren, PhD Professor of Cell Biology at Faculty of Science and Engineering of Åbo Akademi University
	Lea Sistonen, PhD Professor of Cell Biology at Faculty of Science and Engineering of Åbo Akademi University
	Diana Toivola, PhD Docent of Cell Biology at Faculty of Science and Engineering of Åbo Akademi University
Supervised by	John Eriksson, PhD Professor of Cell Biology, Director of Centre for Biotechnology, Chair of Turku Bioimaging, Faculty of Science and Engineering of Åbo Akademi University
	Brian Goodfellow, PhD Auxiliary Professor of the Chemistry Department of Universidade de Aveiro
Reviewed by	Julia Lindqvist, PhD Postdoctoral researcher at Åbo Akademi University

Acknowledgements

Another year has passed, full of emotions, obstacles and memorable moments. To professor John Eriksson, thank you for letting a Portuguese couple into your lab, for believing in us and for all the help. To professor Brian Goodfellow, for the productive feedback delivered during this year. To Julia Lindqvist, for meticulously reviewing this thesis. To Ketlin Adel, for teaching me about flow cytometry and helping me when needed.

To all my friends and colleagues in Turku, for making this stay in this lovely city more pleasant. Arun, "obrigado" for allowing us to crash in your living room and become such a good friend. Alia, "merci" so much for all the patience you had, for all the lessons you gave me, for your friendship, and all the good moments we shared. Your guidance made me a better scientist. Ponnuswammy, thank you for being the most relaxed guy I have ever seen inside a lab. Elnaz, thank you for all your kindness and making us fell home! Alejandro, thank you for all the joy you bring and share. It is impossible to be sad when you are around. Jens, thank you for introducing me into "Battlestar Galactica" and for all the fun moments. Diosa, since you have convinced me to go ice swimming that I secretly hate you, nonetheless, you are my friend, so I forgive you! Maria and Elena, thank you for making this stay in Turku easier by speaking weird Portuguese during our moments together. To all of you, thank you one more time, I know our friendship will still last. My beloved Universidade de Aveiro, it seems that this is the end, thank you for teaching me responsibilities. Thank you to all the teacher I had for guiding me to become a grown and wiser man. Thank you my friends: Antoine, Sofia, Sónia, Danão, Rafa, Calixto, Jackson, Catarina, Marta, "Meireles family", Vivien, Ana, Mariana, for all the good moments we've shared, all the laughter, all the study, all the party, for making Aveiro a perfect city to live, for everything, thank you! To my Erasmus friends, thank you for still keeping in touch. Johannes, I know we don't talk as much, but every time we do, it's like we've never left Odense. You're a true friend, and I hope to see you soon. Thomas, I want my FIFA revenge. We will visit you soon as well, don't worry! My old friends from the small town: Gabriel, Xavier, Robalinho, Rui, Ana Maria, Ana Duarte, Vânia, Inês, thank you for making my weekends at home fun, whenever I didn't have to study and for showing me friendship can really last long distance. A special thank you to Gabriel and Ana Maria, I've know you guys since forever, and yet we remain friend. Thank you for keeping the kid in me alive!

To my family, which made me who I am today! Obrigado pai e mãe, por todo o apoio que me deram nestes últimos anos. Pelo sacrifício que fazem todos os dias. Por me deixarem seguir os meus sonhos, sem nunca colocarem em causa as minhas decisões. Espero que o fim desta minha etapa seja o começo de um período mais calmo para vocês! Podem descansar finalmente, vocês merecem. Irmã, sei que podia escrever em inglês, mas não me apetece. Obrigado por tudo o que fazes por mim. Obrigado por seres a melhor irmã, por me mimares, por me deixares ganhar em todos os videojogos possíveis e imagináveis! Em breve serei eu a mimar-te um bocado, pode ser? Sinto-me em dívida para contigo!

Lastly, I have to thank Leila, my precious girlfriend. Thank you for following all my crazy ideas, even though you're terrified something goes wrong! Thank you for letting me waste my time in videogames even though I should be writing me master thesis. Thank you for all the support, kindness, laughter, for being here always. I might not show it enough, but I really love, and I couldn't be more thankful for having you in my life! You complete me.

Palavras-chave c-FLIP$_L$, proliferação celular, ciclo celular, ciclina E, p27, fosforilação

Resumo

c-FLIP é uma proteína conhecida pela sua capacidade de se ligar ao DISC, onde compete com a procaspase-8 pela interação com FADD. No entanto, existem evidências que a sua isoforma longa consegue regular também o ciclo celular e mecanismos de proliferação. Para além disso, a atividade de c-FLIP$_L$ pode ser controlada por fosforilação. Assim, o objetivo deste estudo é perceber como a fosforilação no resíduo de serina 227 nesta proteína afeta a proliferação e ciclo celular. Neste estudo, observamos que a sobre-expressão de c-FLIP$_L$ com uma mutação de serina para alanina no resíduo 227 levou a uma diminuição da capacidade proliferativa dessas células. O uso de citometria de fluxo permitiu verificar este decréscimo na capacidade proliferativa, assim como uma acumulação de células na fase G1 do ciclo celular aquando da sobre-expressão de S227A c-FLIP$_L$. Os resultados obtidos sugerem que a sobre-expressão de c-FLIP$_L$ controla a população celular através da transição G1/S, através da sua fosforilação no resíduo 227. No entanto, mais estudos são necessários para se perceber a partir de qual mecanismo esta transição é afetada.

Keywords c-FLIP$_L$, cell proliferation, cell cycle, cyclin E, p27, phosphorylation

Abstract

c-FLIP is a protein known for its capacity to bind to the DISC and compete with procaspase-8 for FADD interaction. However, published studies have shown that c-FLIP$_L$ can regulate cell cycle and proliferation. Similarly to many other proteins, c-FLIP can be regulated by phosphorylation. Therefore, the aim of this work was to understand how the phosphorylation of S227 residue on c-FLIP$_L$ affects cell cycle and cell proliferation. We observed that overexpression of phosphodeficient mutant c-FLIP$_L$ lead to a decrease in cell proliferation. Flow cytometric analysis confirmed this decrease, as well as an accumulation of cell at G1 phase of cell cycle, when overexpressing S227A c-FLIP$_L$. Our results suggest that c-FLIP$_L$ overexpression controls cell population size by controlling the G1/S transition, via its phosphorylation. Nonetheless, further studies need to be done to understand which mechanism affects this transition.

Table of contents

List of Tables

List of Figures

Acronyms and abbreviations

ATM	Ataxia-telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
BrdU	Bromodeoxyuridine
CAK	CDK-activating kinase
CamKII	Ca2+ /calmodulin-dependent protein kinase II
Cdc25	Cell division cycle 25
CDK	Cyclin-dependent kinases
c-FLIP	Cellular FLICE inhibitory protein
c-FLIP$_L$	Cellular FLICE inhibitory protein long
c-FLIP$_R$	Cellular FLICE inhibitory protein Raji
c-FLIP$_S$	Cellular FLICE inhibitory protein short
CD95	Cluster of differentiation 95
CFLAR	CASP8 and FADD-like apoptosis regulator
CFSE	Carboxyfluorescein succinimidyl ester
Chk	Checkpoint kinase
Cip/Kip	CDK interacting protein/Kinase inhibitory protein
CKI	Cyclin-dependent kinase inhibitor
CRM1	Chromosomal Maintenance 1
DED	Death-effector domain
DISC	Death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle's medium
DR	Death receptor
ECL	Enhanced chemiluminescence
EdU	5-ethynyl-2′-deoxyuridine
EGF	Epidermal growth factor
E2F	E2 transcription factor
FADD	Fas-associated death domain
FCS	Fetal calf serum
FOXO3	Forkhead box O3
Fucci	Fluorescence ubiquitination cell cycle indicator
GF	Growth factor

G_0	Resting state
G_1 phase	Gap 1 phase
G_2 phase	Gap 2 phase
Hsc70	Heat shock 70 kDa protein 8
IKK	IkB kinase
IL-1β	Interleukin-1 beta
INK4	Inhibitor of cyclin-dependent Kinase 4
KO	Knock-out
Mdm2	E3 ubiquitin-protein ligase
Mik1	Mitotic inhibitor kinase
mRNA	Messenger ribonucleic acid
mTOR	Mamalian target of rapamycin
Myt1	Myelin transcription factor 1
NES	Nuclear export signal
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localisation signals
OptiMEM	Gibco™ Opti-MEM™ I reduced serum media
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PI	Propidium iodide
PKC	Protein kinase C
pRb	Retinoblastoma protein
PTM	Post-translational modification
p15	Cyclin-dependent kinase 4 inhibitor B
p16	Cyclin-dependent kinase inhibitor 2A
p18	Cyclin-dependent kinase 4 inhibitor C
p19	Cyclin-dependent kinase 4 inhibitor D
p21	Cyclin-dependent kinase inhibitor 1
p22-FLIP	FLICE-like inhibitory protein subunit 22
p27	Cyclin-dependent kinase inhibitor 1B
p43-FLIP	FLICE-like inhibitory protein subunit 43
p53	Tumor protein p53

p57	Cyclin-dependent kinase inhibitor 1C
Rel-A	Nuclear factor NF-kappa-B p65 subunit
RIP1	Receptor-interacting protein 1
RIPK	Receptor-interacting protein 1 kinase
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S phase	Synthesis phase
S227	Serine 227
S227A	Serine 227 to alanine mutation
S227D	Serine 227 to aspartate mutation
TNF-α	Tumor necrosis factor alpha
TRAF	TNF receptor associated factors
TRAIL	TNF-related apoptosis-inducing ligand
TRIS	Tris hydroxymethyl aminomethane
v-FLIP	Viral FLICE inhibitory protein
Wee1	Wee1-like protein kinase
WT	Wild type

1 Introduction

The entire multicellular organism originates from a single cell, which divides and leads to an increase in cell number. Then, these cells receive different signals to differentiate. Proliferation and differentiation are two key processes required to build a functional organism.

Although proliferation and differentiation are essential, the organism needs to remove all unwanted and damaged cells that appear continuously. This is done by death-inducing signals, that lead to cell death. Cell proliferation and cell death are processes that need constant regulation to be kept in equilibrium. This helps the organism to avoid physiological complications that might be life-threatening.

The cell cycle can also control the balance between life and death of a cell. This control depends either on changes in the environment or signals that reach the cells. How the cell interprets this can cause cell cycle arrest or cycle progression until a cell divides into two cells.

2 Review of the literature

2.1 Cell cycle

The cell cycle is a process dependent on a series of steps, which culminate in the division of a single cell into two daughter cells. The cell cycle is divided into three stages: interphase, mitosis and cytokinesis. In between or during these stages, there are also checkpoints, which allows the organism to check if cell division is being performed correctly (1).

Before undergoing division, the cell needs to prepare itself by producing proteins and duplicating its deoxyribonucleic acid (DNA) content. This is done during interphase, which is divided into three phases: two gap phases (G_1 and G_2), and a synthesis phase (S) in between the G phases (figure 2.1). The G phases are both related with growth and preparation of the cell for the following steps. If the cell senses that it is not prepared for division during G_1 phase, or the environment is not ideal, this phase can be prolonged, or the cell can enter a special resting state, known as G_0 (2). When conditions are favourable, the cell progresses to the S phase, a crucial step where DNA replication occurs. This stage is controlled by replication protein A (RPA), which interacts with single strand DNA, signalling a checkpoint, which ensures that the genetic material is correctly duplicated, before the cell

proceeds to mitosis (3). During G2, the cell continues to grow and producing proteins needed for division, and DNA condensation starts (4).

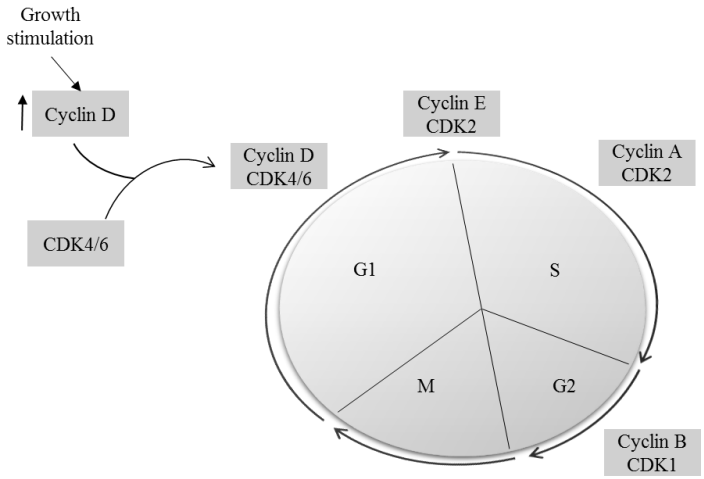

Figure 2.1 Cell cycle. The cell cycle begins when the cell is stimulated by a growth factor, which causes increase of cyclin D, that complexes with either CDK 4 or 6, and the cell progresses through G1 phase. G1/S transition requires activity of cyclin E-CDK2 complex, whereas cyclin A-CDK2 complex allows cell progression through S phase. Lastly, cyclin B-CDK1 complex are important for mitosis entry.

Mitosis is composed of different phases: prophase, prometaphase, metaphase, anaphase and telophase, which prepare the cell for division. During this stage, the replicated DNA will continue to condense. The chromosomes will then bind to the formed mitotic spindle, aligning them at the equator of the cell. Before proceeding with separation of the sister chromatids, the cell checks if the spindle is well attached to the chromosomes, making sure that the division will be even. If everything is under control, the sister chromatids are separated, each of them reaching an opposite pole of the cell. Finally, the cell starts the division of the cytoplasm. The creation of two daughter cells is achieved at the cytokinesis stage (1).

Since cell cycle is such an important part of life, it needs to be carefully controlled. For this to be accomplished, several proteins suffer changes in their levels or activity along the cycle, allowing progression through different cell cycle phases.

2.1.1 Regulators of cell cycle

The cyclin-dependent kinases (CDKs) are included in a family of serine/threonine protein kinases. There are various types of CDKs, but only four are activated during cell cycle and their activity is restricted to specific points of cell division (5). For instance, CDKs 4 and 6

are activated at the G_1 phase, CDK2 at G1 and S phase and CDK1 at mitotic phase (6). While active, CDKs can phosphorylate several targets. However, to be activated, these enzymes need to interact with activating proteins, known as cyclins. This association leads to formation of cyclin-CDK complexes, which are cell cycle stage specific (5). Although CDK levels are steady in the cell throughout cell cycle, the levels of cyclin change in a cyclical way, except for cyclin D, which expression needs growth factor stimulation (7). There are four different cyclins that can interact with CDKs to control cell cycle progression (figure 2.1). Cyclin D interacts with CDKs 4 and 6, forming a complex that controls entry to G_1 (8). Cyclin E will interact with CDK2 and controls progression from G_1 into S phase (9). Cyclin A is needed during S phase and interacts with CDK2 (10). Finally, cyclin B, which is necessary for mitosis entry, interacts with CDK1 (figure 2.1) (11). When cyclins are no longer needed, they are marked for proteolysis by ubiquitin addition (12).

Although cyclins are the major regulators of CDKs, CDKs are also regulated by phosphorylation on conserved threonine and tyrosine residues. CDKs 1, 4 and 2 require phosphorylation at a specific threonine to be fully activated (13). This phosphate addition is done by CDK-activating kinase (CAK) (14). There are also negative regulators of CDKs, such as Wee1-like protein kinase (Wee1) and myelin transcription factor 1 (Myt1) kinases, which is are kinase that phosphorylates CDKs to block their activity. However, this phosphorylation can be removed by a phosphatase known as cell division cycle 25 (Cdc25) (15).

Other negative regulators of cell cycle are CDK inhibitors (CKIs). They can bind to either cyclin-CDK complex or to CDK alone, regulating their activity. Two different CKI families have been identified: the inhibitor of Cyclin-Dependent Kinase 4 (INK4) family and the CDK interacting protein/Kinase inhibitory protein (Cip/kip) family (6). The INK4 family includes p15, p16, p18 and p19, which specifically bind and inactivate CDK4 and CDK6 (16). This occurs before cyclin binding, preventing this association to happen (16). The second family of CKIs is composed of p21, p27 and p57 (17). This CKI family is able to inactivate all cyclin-CDK complexes (18,19). Besides this function, cyclin-dependent kinase inhibitor 1 (p21) can also inhibit DNA synthesis.

2.1.2 Control of cell division and cell growth

Mitogens are a group of proteins that include several growth factors (GFs), such as epidermal GF, platelet-derived GF, among others. This group of proteins can stimulate many types of cells to divide by acting in the G_1 phase of the cell cycle (20). Entry to G_1 phase depends on the formation of cyclin D-CDK4, or -CDK6, complexes. However, production of cyclin D only occurs upon mitogen stimulation (21). When a mitogen associates with a cell membrane receptor, it will activate a signalling pathway, which increases the expression of immediate early genes. Among these, there is one that encodes for c-Myc, a transcription factor, which increases the expression of delayed-response genes (1). Cell cycle progression is dependent on a series of positive feedback loops that control cyclin-CDKs levels, making sure the cell is prepared to complete the division process (22). Besides mitogens, cells are also stimulated by other growth factors, which stimulate cell growth, but not cell division. During cell division, the cell is also a target of survival factors, which supress apoptosis.

2.1.2.1 G1/S transition

The cell cycle progression not only depends on extracellular signals, but also requires dynamic changes in gene expression. These changes are controlled by CDK activities, which in turn influence cyclin levels. The cell cycle has three known transcriptional waves, which coincide with the cell cycle transitions (23). The first transcriptional wave occurs before G1/S and depends on the E2 transcription factor (E2F) family of transcription factors, together with DNA binding partners and pocket proteins, such as retinoblastoma protein (pRb) (24). These pocket proteins are important for cell cycle through interactions with E2F transcription factor (25). At early G1 phase, activator E2F proteins are bound and inhibited by pRb, repressing E2F activity at the promotor region of the gene. Upon phosphorylation of Rb by cyclin D-CDK4, E2F is released and can initiate transcription.

Upon G1-S transcriptional activation, an “all-or-none switch” is created, which forces the cell to enter the cell cycle. This point is known as the restriction point, and it is characterised by increased cyclin-CDK activity and phosphorylation of pocket proteins. This allows initial activation of transcription of cyclin E. This activation is further increased by cyclin D, which increases its own transcription through positive feedback mechanisms (26). This causes accumulation of cyclin E and A, which then form a complex with CDK2, leading to its

activation. Upon G1/S transcription initiation and CDK2 activation, the cell progresses to S phase, where DNA replication starts.

For cyclin/CDK activation, cyclin-dependent kinase inhibitor 1B (p27) and p21 have to be degraded to avoid its inhibition (17). It was recently suggested that p27 is the molecule responsible for inhibiting G1/S transition, and so, its abrupt degradation ensures S phase entry (27). Due to this, the activity of cyclin E/CDK2 must be high enough to overcome p27 inhibition and mark this CKI for degradation (27).

Once the cell has entered S phase, transcription must be inactivated, which is accomplished by negative feedback loops (figure 2.2). It has been proposed that CDK activity inactivates E2F-mediated transcription, since E2F1, member of the E2F family, is bound and phosphorylated by cyclin A-CDK2 (figure 2.2) (28). This promotes E2F1 dissociation from DNA, and inactivation of its gene targets. Since the gene that encodes cyclin A is a target of E2F, this constitutes a negative feedback loop (28). Additionally, cyclin A-CDK2 activity is inhibited by p27, which is targeted for degradation by cyclin E-CDK2 and cyclin A-CDK2. Thus, cyclin A-CDK2 inhibit E2F1 activity, which will no longer stimulate cyclin A and E genes, decreasing their protein production (29). Furthermore, protein levels of cyclin A and E will decrease due to degradation by p27 (30).

Among the E2F family, there are some proteins that act as transcriptional repressors (31). These have been suggested to be part of additional negative feedback loops, which are important to inactivate G1/S transcription (32).

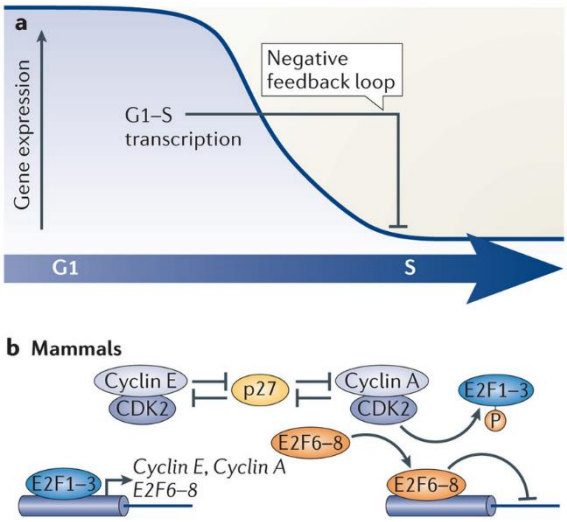

Figure 2.2 G1/S transition. a) When the cell enters S phase G1/S transcription must be inactivated, which involves negative feedback loops. **b)** p27 is targeted for degradation by both cyclin A-CDK2 and cyclin E-CDK2. Higher activity of cyclin A-CDK2 results in phosphorylation of E2F1, which inactivates transcription. Adapted from Bertoli et al. 2013 (33).

2.1.2.2 G2/M transition

As referred to previously, cell cycle progression is controlled by CDKs. The activity of these kinases is controlled by Cdc25, an activator of CDKs, and Wee1, which is an inactivator of CDKs. At the G2/M border only CDK1 is active. For this kinase to be active, it needs to associate with cyclin A or B, in animal cells (11). These cyclins accumulate during previous cell cycle phases, and are degraded when the cell reaches metaphase, which in turn causes inactivation of CDK1 (12). The cyclin B-CDK1 complex is actively kept in the cytoplasm, during G2 phase, making it unable to phosphorylate its nuclear targets. This is achieved by binding of the cyclin subunit to an exportin protein called chromosomal maintenance 1 (CRM1) (34). In turn, the import of the complex is done by importin β, also by interaction with the cyclin subunit (35). For the complex to be imported, cyclin B needs to be phosphorylated in its CRM1-binding site, blocking its binding to the exporter, allowing complex accumulation in the nucleus. The complex is kept inactive by two inhibitory phosphorylations catalysed by Wee1 and Myt1 (36). To activate CDK1, the inhibitory phosphorylations have to be removed by Cdc25 phosphatase (37). Additionally, CDK1 needs to be phosphorylated by CAK, achieving its active state (38). This whole mechanism is further activated by a positive feedback loop involving Cdc25, which is phosphorylated by Cdk1, leading to higher Cdc25 activation (37). This mechanism ensures that the G2/M transition is completed, and the cell enters the mitosis phase.

2.1.2.3 Cell cycle arrest

Even though cell division is meticulously controlled, errors still occur during the process. One of the most important errors are related to DNA damage that might arise during replication, in the S phase. Nevertheless, the cell cycle has checkpoints, which are regulatory mechanisms that ensure that the cell cycle occurs without problems. These checkpoints occur during G1/S transition, G2/M transition and before anaphase. DNA is the major target during these checkpoints. If DNA damage is sensed by ataxia-telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3-related (ATR), the cell cycle is arrested until the damage is repaired (39). Studies have shown that CDK1 is an important effector during G2 checkpoint. Upon DNA damage, ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) protein kinases are recruited to the damaged site, and phosphorylate checkpoint kinase 1 (Chk 1) and checkpoint kinase 2 (Chk 2) (figure 2.3) (40). Chk1 is

required for cell cycle arrest due to damaged DNA, while Chk2 is required in response to unreplicated DNA (41,42), and both can phosphorylate Cdc25 (43). This phosphorylation creates a binding site for proteins of the 14-3-3 family, which sequester Cdc25 in the cytoplasm, leaving it unable to dephosphorylate CDK1 (44,45). However, other studies suggested that CDK1 is not the only protein necessary for mitotic delay (46). In this case, mitotic inhibitor kinase (MIK1) is an important target to induce delay. Overexpression of Chk1 was shown to cause MIK1-dependent mitotic arrest, suggesting MIK1 to act downstream of Chk1 (46).

Another target of Chk1 and Chk2 is the tumour suppressor p53, which has the ability to induce either cell cycle arrest or cell death. As a response to stress, p53 is phosphorylated and activated (figure 2.3) (47). Typically, p53 is found at low levels in unstressed cells, since it is targeted for degradation by E3 ubiquitin-protein ligase Mdm2 (48). However, upon DNA damage, ATM and ATR will phosphorylate p53 (49). Therefore, Mdm2 will no longer mark p53 for degradation, leading to p53 accumulation and consequent activation of its target genes (50). Studies in human cells lacking p53 showed that G2 arrest still occurs. This shows that there are other pathways to arrest G2 phase that do not require p53 (51). On the other hand, overexpression of p53 leads to cell cycle arrest in G2 phase, even though the cells were not stressed (51).

When p53 regulates G2 arrest, it activates genes that induce apoptosis, along with genes that induce growth arrest. Its major target is p21 gene, which is important for G1 arrest (52). When its transcription is activated, p21 production starts, thus blocking entry into the cell cycle, because this is a CKI that targets both cyclin E-CDK2 and cyclin A-CDK2 complexes (figure 2.3) (53). Although this process occurs during S phase, it can affect G1/S transcription. It was suggested that, under replication stress, Chk1 phosphorylates E2F6, inactivating it, which allows G1/S transcription to continue (54). G2 arrest is not achieved by p21 activation, since this protein binds poorly to CDK1 (55). However, studies have shown that p53 can induce transcription of other genes, such as GADD45 (56). The produced protein was shown to bind to CDK1 and to dissociate cyclin B-subunit, inactivating the complex (56). Other studies were performed to understand how p53 affects cyclin B1-CDK1 complex (57,58). It was suggested that p53 may alter the cytoplasmic/nuclear shuttling of cyclin B1 to inhibit cyclin B1-CDK1 complex, however without any direct evidence (59). Several groups have reported that maintenance of G2 arrest after DNA damage requires

transcriptional repression mediated by Rb proteins (60,61). Among these studies, it was suggested that one of pRb targets during p53-dependent G2 arrest is CDK1 (61). There are several mechanisms to ensure that no errors remain in the cell. However, during cell cycle arrest, sometimes the damage is so severe that the repair mechanisms are unable to overcome it, and the cell undergoes apoptosis.

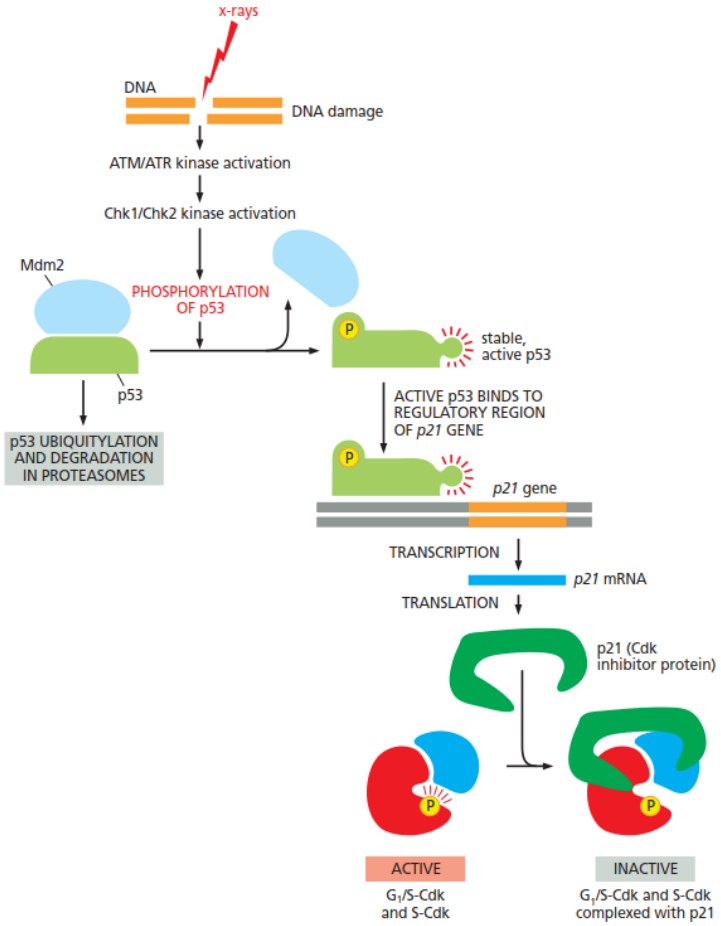

Figure 2.3 Cell cycle arrest by DNA damage. DNA damage causes ATM/ATR activation, followed by Chk1/Chk2 activation. This causes p53 activation, which binds to p21 gene, stimulating p21 protein production. Active p21 binds to cyclin E-CDK2 and cyclin A-CDK2, arresting cells in G1 (1)

2.2 Cellular FLICE inhibitory protein (c-FLIP)

2.2.1 Isoforms

FLIP was first identified while searching for death-effector domain (DED)-containing proteins that could regulate apoptosis (62). It was named as viral-FLIP (v-FLIP), since six different FLIP were found in γ-herpesvirus and molluscipoxvirus (63). It was shown that the protein turns infected cells resistant against cluster of differentiation 95 (CD95) and TNF-related apoptosis-inducing ligand (TRAIL) receptor induced apoptosis (64). This is achieved

because v-FLIPs have two DEDs, which can bind to Fas-associated death domain (FADD), an adaptor protein that associates with the death receptor. This interaction interferes with the interaction of FADD with procaspase-8, an inactive initiator caspase needed for apoptosis signalling, and inhibits procaspase-8 activation (64).

Shortly after the discovery of v-FLIPs, its mammalian homologue was described and termed c-FLIP (65). Three different isoforms were discovered: c-FLIP long (c-$FLIP_L$, 55kDa), c-FLIP short (c-$FLIP_S$, 27kDa) and c-FLIP Raji (c-$FLIP_R$, 25kDa) (figure 2.4) (65,66). Interestingly, all these isoforms are encoded by CASP8 and FADD-like apoptosis regulator (*CFLAR*) gene and generated from alternative splicing (66). Even though each isoform undergoes different processing, their two DEDs are similar. Compared to the other c-FLIP isoforms, the long one has an additional caspase-like domain, which is catalytically inactive due to replacement of a cysteine residue within the Gln-Ala-Cys-X-Gly-motif and a histidine within the His-Gly-motif (67). The short isoforms have a short C-terminal tail of 19 and 17 amino acids for c-$FLIP_S$ and c-$FLIP_R$, respectively (66).

Since c-FLIP has in its structure DEDs, it competes with procaspase-8 for FADD interaction at the death-inducing signalling complex (64). Although v-FLIP and c-FLIP have been shown to have anti-apoptotic properties, some studies have shown that the long form can act as an activator of apoptosis.

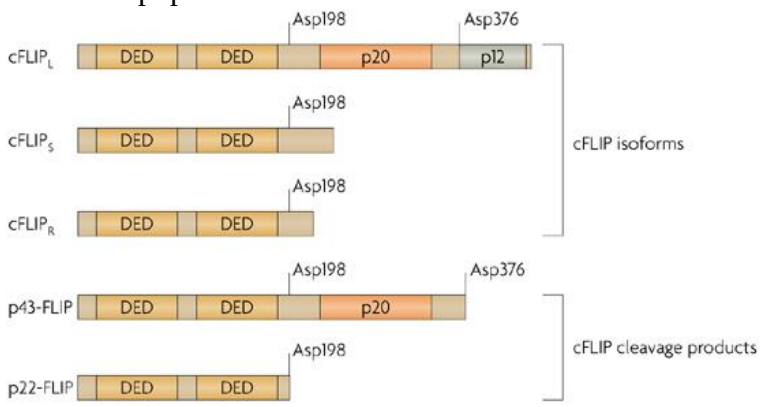

Figure 2.4 c-FLIP isoforms. Schematic representation of all c-FLIP isoforms and cleavage products. p20 and p12 are the inactive caspase-like domains of c-$FLIP_L$. Asp198 and Asp376 are the cleavage sites, which lead to generation of p22-FLIP and p43-FLIP, respectively (68).

2.2.2 Regulation of protein levels

The functions of c-FLIP in the cell are highly dependent on their cytoplasmic protein levels (69). Its levels can be controlled in several ways: protein synthesis, protein degradation, posttranslational modifications (PTMs) and subcellular localisation (65,70–75).

Protein synthesis begins with a gene being transcribed into a messenger RNA (mRNA), which is in turn translated into a polypeptide and folded into a specific conformation, producing a mature protein. Although c-FLIP is constitutively expressed in different normal cells, its gene expression can be controlled by a number of stimuli (76). The *CFLAR* gene expression is enhanced by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p53, for instance, causing higher amount of protein synthesis (70,77). On the other hand, *CFLAR* transcription is supressed by c-Myc and forkhead box O3 (Foxo3) (72,78). So, depending in which signals reach the targeted cell, c-FLIP can be produced in higher or lower levels than normal.

Proteins can be marked for degradation, another process that helps controlling protein amount. This process is carried out by the 26S proteasome, hydrolysing the targeted protein into smaller polypeptides, which are then released and reused by the cell, if needed. It was shown that c-FLIPs is degraded by this mechanism (71). In addition, c-FLIP$_S$ has been proven to have a shorter half-life that c-FLIP$_L$. This different stability between c-FLIP isoforms has been associated with differences between their C-terminal region. c-FLIP$_S$ has two crucial lysines in its C-terminal tail, which makes it more sensitive proteasomal degradation, when compared to other isoforms (79).

PTMs can also affect c-FLIP levels. PTMs are achieved by addition of a molecule or small protein onto the target protein, which will have distinct outcomes. Ubiquitination is a PTM which marks a protein for degradation (80). The added protein is named ubiquitin, and it bind to lysine residues of the target molecule. Ubiquitin can form chains, or it can be added as a monomer (81). After addition of a ubiquitin chain to a lysine residue in a three-step enzyme cascade, a recognition step is performed by ubiquitin receptors. These receptors are able to distinguish different kinds of ubiquitin chains, which can lead to different outcomes in cells (73). As mentioned before, c-FLIP$_S$ has two lysines (K192 and K195) in its C-terminal region. These lysines make it more sensitive for proteasomal degradation, since ubiquitin is added to these residues (79). It was also shown that c-FLIP$_L$ can be degraded upon tumour necrosis factor alpha (TNF-α) stimulation by the E3 ligase Itch, an enzyme needed for ubiquitin addition (82). Also, c-FLIP$_S$ was shown to be ubiquitinated when mamalian target of rapamycin (mTOR) complex 2 is inhibited (83).

Besides ubiquitination, there are other PTMs. Protein phosphorylation and dephosphorylation are one of the most important PTMs in signalling pathways mediated by

external signals. Phosphorylation of a protein means addition of a phosphate group into one of these three hydroxyamino acids: serine (S), threonine and tyrosine. This reaction is achieved by transfer of a terminal phosphate group from ATP to the amino acid side chain of the target residue, and it is catalysed by protein kinases (84). Phosphorylation causes conformational changes in the modified protein, which can, for example, increase or decrease its activity or ability to interact with other molecules (85). To oppose protein kinase activity, multicellular organisms have protein phosphatases, which are able to remove a phosphate group from proteins, altering the target protein activity or interactions (86). c-$FLIP_L$ can be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), promoting its recruitment to the death-inducing signalling complex (DISC) (74). Protein kinase C (PKC) phosphorylates c-FLIPs at S193, a common phosphorylation site of all c-FLIP isoforms. Due to the proximity of S193 to K192 and K195 in c-$FLIP_S$, it was explored if these PTMs would influence each other. It was shown that disruption of S193 phosphorylation increased ubiquitination, affecting the half-lives of the short isoform (87). Also, S273 was reported to be phosphorylated by PI3K/Akt, resulting in degradation of c-$FLIP_L$ during macrophage activation (88).

The cell is divided in several compartments, and so, they can be used to store proteins in one place, changing their local concentration and kinetics. c-FLIP activity is believed to be controlled by changes in its subcellular localisation (75,89). Although it was thought that c-FLIP was only localised in the cytoplasm due to its anti-apoptotic function, it was shown that it can also be expressed in the nucleus, because c-$FLIP_L$ has in its amino acid sequence two nuclear localisation signals (NLSs) and a nuclear export signal (NES) (75,89). The NLSs was shown to be important for c-FLIP nuclear localisation, because mutated NLS leads to no c-FLIP in the nucleus (75,89).

Another way of c-FLIP level control is by proteolysis. This is achieved by proteases, such as caspases, and they are able to cleave c-FLIP into smaller fragments (90). This occurs because c-$FLIP_L$ has two aspartic cleavage sites, D196 located between DED2 and the caspase-like domain, and D376 in the middle of the caspase-like domain (65,91). The cleavage is done by caspase-8, after its activation and interaction with c-$FLIP_L$ (65). When cleaved at D376, FLICE-like inhibitory protein subunit 43 (p43-FLIP) is produced, but only from c-$FLIP_L$ (figure 2.4). Cleavage at the D196 residue produces FLICE-like inhibitory

protein subunit 22 (p22-FLIP) (figure 2.4), which can be obtained from all c-FLIP isoforms, which can induce NF-κB activation (91,92),.

2.2.3 Functions of c-FLIP

2.2.3.1 Apoptosis

A tight control of c-FLIP is required, since this protein can affect different cell processes. When it was first discovered, FLIP was associated to apoptotic functions as an inhibitor of death receptor (DR)-mediated apoptosis. However, recent studies showed that it has functions not related to apoptosis. In order to inhibit DR-mediated apoptosis, c-FLIP needs to be recruited to the DISC via DED interactions (65). The short isoforms of c-FLIP, when bound to the DISC, are able to inhibit procaspase-8 activation and block CD95- or TRAIL-induced apoptosis (65,91). Even though this function of the short isoform is clear, c-$FLIP_L$ function in apoptosis lacks consent. It was shown that the long isoform can have anti-apoptotic function, similar to c-$FLIP_{S/R}$, when expressed at high levels (91). However, endogenous expression of c-$FLIP_L$ makes it act as a pro-apoptotic molecule (93). This is mediated by formation of a procaspase-8/c-$FLIP_L$ heterodimer, which results in partial cleavage of procaspase-8. Therefore, in this situation, instead of the usual two cleavages, only the first one is completed (94). This results in the formation of a p10 fragment and a partially active p43-caspase-8 fragment. Interestingly, procaspase-8/c-$FLIP_L$ heterodimerization occurs with more affinity than procaspase-8 homodimerization, allowing a tighter regulation of caspase-8 activation at the DISC (95).

The p43-caspase-8 fragments have been shown to be unable to initiate death signalling, but they can activate themselves and receptor-interacting protein 1 kinase (RIPKs) (90). Although this is not enough to initiate apoptosis, this fragment is able to cleave RIPKs. This is useful in TNF-α-mediated apoptosis, since c-FLIP can regulate formation of either complex IIa, which leads to apoptosis, or complex IIb, which leads to necroptosis (96). When caspase-8 activation is inhibited, complex IIb is preferably formed, allowing activation of necroptosis due to accumulation of RIPK1 and RIPK3. Nonetheless, with this partial activity of p43-caspase-8 molecule, these RIPKs will be cleaved, which impairs activation of necroptosis (91).

2.2.3.2 Proliferation

Although the short isoforms of c-FLIP are only related to anti-apoptotic functions, c-$FLIP_L$ has functions beyond regulation of DR-mediated apoptosis, and are related to cell survival and cell proliferation. Survival and cell proliferation functions are achieved by p43-FLIP, which is a fragment obtained from c-$FLIP_L$ cleavage at D379 by caspase-8 (90).

Although caspase-8 is mainly known for its role as an initiator caspase at DR-mediated apoptosis, it has been reported to have different functions outside the apoptotic process. Caspase-8 can, for instance, initiate NF-κB activation independently of its proteolytic activity (97). It can also regulate cell migration due to interaction with integrins, or modulate interleukin-1 beta (IL-1β) production upon macrophage activation (98). Since c-FLIP can regulate caspase-8 activation at the DISC, it might have an indirect influence on the different pathways regulated by DISC. However, more studies are needed to understand the role of c-FLIP in these different caspase-8 functions.

The NF-κB protein family is a family of transcription factors, which are responsible for inflammation, immune response, protection against apoptosis and differentiation (99). When NF-κB is free it can translocate into the nucleus, allowing transcription regulation of anti-apoptotic and pro-survival proteins (100). It was recently shown that p43-FLIP can interact with TNF receptor associated factor 1 and 2 (TRAF1 and TRAF2) and receptor-interacting protein 1 (RIP1), promoting activation of NF-κB (90,101). Additionally, another fragment obtained from c-FLIP cleavage at D196, p22-FLIP, can interact with IKKγ subunit of the IkB kinase (IKK) complex and stimulate activation of this signalling pathway (92). This fragment differs from p43-FLIP, since it can be produced without DR stimulation (92,102). Besides its functions in DR-induced apoptosis, c-$FLIP_L$ also regulates DR-induced NF-κB activation (103). This occurs in a concentration-dependent manner. It was shown that high amounts of c-$FLIP_L$ in the cell, will cause more association of c-$FLIP_L$ to the DISC. Subsequently, less procaspase-8 will be recruited, forming less procaspase-8/c-$FLIP_L$ heterodimer. Further, formation of p43-FLIP will be reduced, directly affecting NF-κB activation. Nevertheless, studies showed that moderate levels of c-$FLIP_L$ are needed for DR-induction of NF-κB pathway (103–105).

2.2.3.3 Cell Cycle

Studies using c-FLIP$_L$ overexpression have suggested that this protein also induces p27 depletion (106). Additionally, Quintavalle et al. have shown that the p27 mRNA levels were also reduced in these cells (106). As referred before, p27 is a CKI that inhibits cyclin E-CDK2 and cyclin A-CDK2 complexes (figure 2.5) (18). Since, in this case, p27 is decreased, it is possible that the concentration of cyclin A and E are increased. Also, p27 was suggested to be the molecule responsible for inhibiting G1/S transition (27). Thus, if p27 levels are decreased upon c-FLIP overexpression, the G1/S transition will no longer be inhibited, which could make this transition process faster. Further, it was shown that Fas/FADD/c-FLIP$_L$/caspase-8 complex control G1/S transition in epidermal growth factor (EGF)-stimulated hepatocytes (107). In this study, it is suggested that c-FLIP$_L$ expression is induced by EGF stimulation, thus increasing the levels of c-FLIP bound to FADD (107). At FADD, c-FLIP$_L$ associates with procaspase-8, causing cleavage of both proteins and, subsequently, higher levels of p43-FLIP (107). Activation of NF-κB by c-FLIP$_L$ might also have effects on cell cycle progression. Studies have shown that NF-κB has three binding sites in the cyclin D$_1$ promoter (108). The sites mainly bind NF-κB$_1$/Rel-A complex, which is increased upon mitogen stimulation (93,94). Thus, when mitogens promote cell division, it promotes cyclin D$_1$ activation by NF-κB, stimulating G$_1$/S phase transition (figure 2.5).

The main functions of c-FLIP depend on its cleavage or DED-dependent interactions with other proteins. Like many other proteins, c-FLIP is regulated by phosphorylation. However, little is known whether this type of PTM plays an important role in its functions. Additionally, little is known about how does c-FLIP influence cell population, and if a phosphorylation site would be the needed for this mechanism.

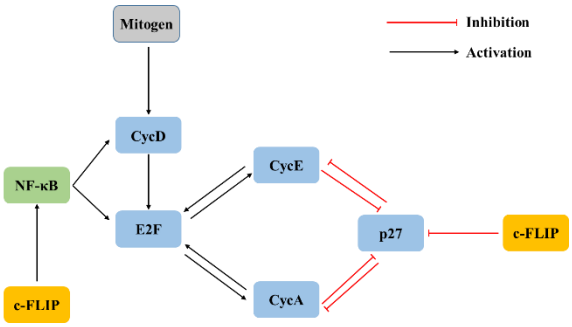

Figure 2.5. c-FLIP interacts with molecules that regulate cell cycle. c-FLIP activates NF-κB, which in turn is known to activate cyclin D, stimulating G1/S transition. Additionally, c-FLIP was found to stimulate decrease of p27 levels. p27 inhibits cyclin E and A, important regulators of G1/S transition.

2.3 Flow cytometric cell proliferation dynamics

Flow cytometry is a technology that can measure optical and fluorescence characteristics of a single cell, as it flows in suspension through a light source. It can analyse the size and granularity of cells, as well as their fluorescent features, which is derived from either dyes or antibodies (111). When a cell is labelled with a fluorescent probe, the fluorescence that the instrument detects is proportional to the amount of probe bound to the cell or cellular component.

This technique is used in several applications, to detect whole cells or cellular components. These components can help studying cell proliferation and cell cycle, among other mechanisms of the cell (112,113). In this thesis, the focus will be on cell proliferation and cell cycle methods.

2.3.1 Carboxyfluorescein succinimidyl ester (CFSE)

Labelling cells with CFSE is commonly used for cell proliferation studies. To perform this label, cells are kept in medium rich in a CFSE precursor molecule, which diffuses into the cell. Once inside, it is converted into fluorescent CFSE and binds to proteins, which makes the dye unable to pass through the cell membrane. Subsequently, the medium is changed to remove extra CFSE precursor molecules. After labelling the cells, the sample is analysed at sequential time points using flow cytometry, by measuring the fluorescent intensity of the individual cells (figure 2.6) (112). The fluorescent intensity is proportional to the amount of label bound to cellular proteins and autofluorescence. Thus, the decrease in intensity can either be due to cell division or degradation of cells labelled with CFSE, since CFSE is degraded with the protein. Additionally, if a cell dies, CFSE is degraded, so these cells do not contribute for the fluorescence (114). After a certain number of cell divisions, the fluorescence will become undistinguishable from the autofluorescence. From the obtained data it is possible to learn how many cell divisions cells undergo (115).

2.3.2 Click-iT 5-ethynyl-2′-deoxyuridine (EdU)

Another method to access cell proliferation is measuring DNA synthesis. This was initially performed by measuring incorporation of radioactive nucleosides. However, this technique was subsequently replaced with antibody-based detection of bromodeoxyuridine (BrdU) (116). This method has several limitations and requires a combination of acid, heat,

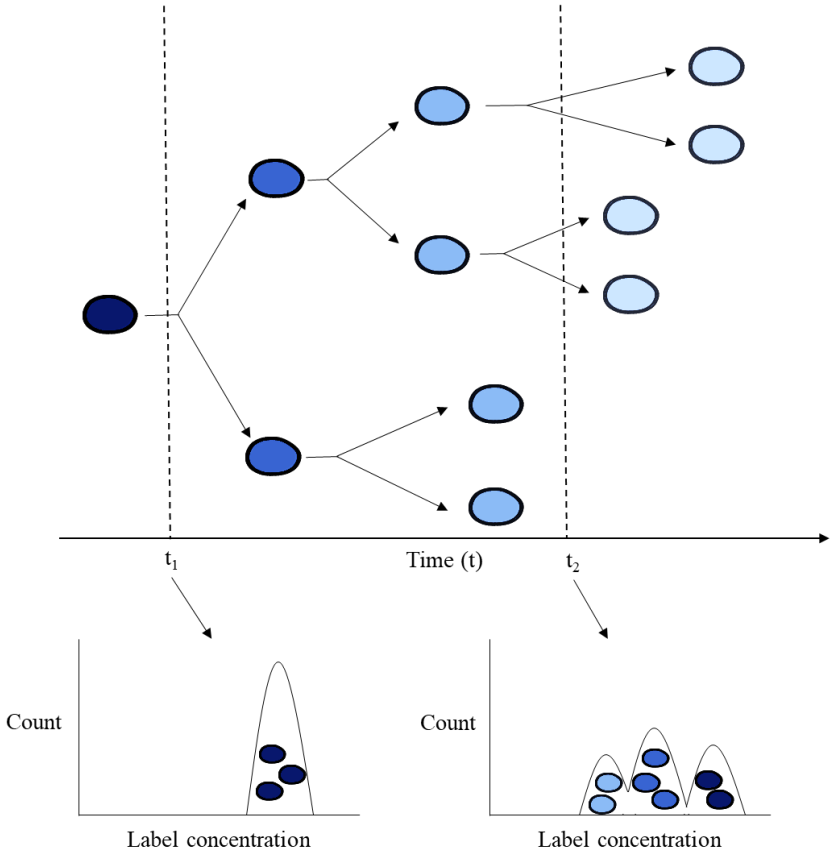

Figure 2.6. Illustration of CFSE dilution due to cell division. Cell division results in halving of the concentration at each division (top). Cells do not divide or proliferate at the same rate, thus we can observe different label concentration distribution within the same population (bottom). Two time points are depicted by vertical lines, which result in different fluorescence distribution, with t_1 yielding only one peak, while t_2 shows a mixture of different generations.

or nucleases treatments to allow the antibody to access the incorporated BrdU residues. Thus, new methods were developed over the years to improve detection of S-phase cell cycle progression. EdU, which is a nucleotide analogue, is found to be incorporated into DNA via click reaction, which enhances the detection of S-phase cell cycle progression (117,118). With this method, the DNA is not subjected to harsh treatments, maintaining its helical structure, allowing cell cycle staining or labelling of cell surface markers. After EdU treatment the cells are fixed, permeabilised and click-labelled. The fluorescence readout can be performed using flow cytometry, which gives a histogram with two peaks, one with non-proliferating cells and another with proliferating cells, which are labelled with EdU.

Additionally, a staining with propidium iodide (PI) can be performed simultaneously with EdU. PI is a fluorescent intercalating agent used to stain cells in flow cytometry to evaluate either cell viability or cell cycle (119,120). For cell cycle analysis, the cells must be fixed to allow PI to bind to DNA. This dye is stoichiometric, meaning that the binding is proportional

to the amount of DNA. The G2 population will fluoresce more brightly, since it has double the DNA content, while G1 phase will present the lowest fluorescence. S phase population, in its turn, will present a population between G1 and G2 phase, since it will take up the fluorescence while the DNA is replicating (119).

Staining cells with EdU and PI, results in a dot plot with a horseshoe shaped form, which tells not only if the cells are proliferating, but also in which cell cycle phase the cells are in. From this, we can understand how the cell cycle is influenced under the studied circumstances.

3 Aims and outline

c-FLIP is a protein known for its inhibitory role in DR-mediated apoptosis, through regulation of procaspase-8 activation at the DISC. Studies have reported that c-FLIP can be phosphorylated by PKC at serine 193 (S193) and at serine 227 (S227). However, little is known about their role in c-FLIP$_L$ functions. It was also suggested that c-FLIP overexpression can decrease p27 protein and mRNA levels. With this decrease in both mRNA and protein levels, the remaining amount of p27 will not be as effective to inhibit cyclin A-CDK2 and cyclin E-CDK2. Unpublished data collected in our lab suggested that a serine to alanine mutation at residue 227 of c-FLIP$_L$ causes the cells to proliferate less when compared to cells transfected with the empty plasmid vector. Furthermore, overexpression of c-FLIP$_L$ wild type (WT) makes the cells grow faster than cells transfected with an empty vector. Therefore, it was hypothesised that either S193 or S227 phosphorylation of c-FLIP$_L$ could regulate cell cycle and cell population size. To do so, a series of flow cytometric studies were performed to infer if c-FLIP$_L$ overexpression leads to faster proliferation of cells. Further, flow cytometry was used to study differences of cell cycle progression between cells transfected with mock, c-FLIP$_L$ WT, serine 227 to alanine mutation (S227A) and serine 227 to aspartate mutation (S227D). Regulators of G1/S transition were also studied to study differences in protein levels between different samples.

4 Materials and methods

4.1 Cell culture

HeLa and fluorescence ubiquitination cell cycle indicator (Fucci) HeLa cells were cultured in a humidified 5% CO_2 atmosphere at 37ºC in Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 4500 mg/L glucose, 10% fetal calf serum (FCS), antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), 2 mM L-glutamine and passaged when they were 80 % confluent.

Fucci HeLa cells express Fucci technology. The Fucci technology allows easy determination of G1 and S/G2/M phases of the cell cycle with fluorescent probes: G1-orange and S/G2/M-green. The fluorescence is controlled by ubiquitin-mediated proteolysis, which degrade the probes in a cell-cycle dependent manner. The green probe is degraded during G1 phase, while the orange one is degraded at S/G2/M phases.

4.2 CFSE staining

8×10^6 HeLa or Fucci HeLa cells were centrifuged and resuspended in 1 mL of Phosphate buffered saline (PBS), and 5 µL of CellTrace™ Violet staining solution (Invitrogen) was added. Cells were incubated for 20 minutes in a humidified 5% CO_2 atmosphere at 37ºC, protected from light. 5 mL of DMEM was added to the cells, followed by 5 more minutes of incubation. Cell were centrifuged and resuspended in pre-warmed Gibco™ Opti-MEM™ I Reduced Serum Media (OptiMEM) (GibcoBRL) for transfections.

4.3 Transfections

For transfections, 8×10^6 Fucci HeLa and HeLa were centrifuged and resuspended in 1.6 and 2 ml of OptiMEM, respectively, and 0.4 mL was added to electroporation cuvettes (BTX) with pre-added 10 µg of plasmid DNA. Cells were subjected to a single electric pulse (220 V, 975 µF) in 0.4 cm electroporation cuvettes using a Bio-Rad Gene Pulser electroporator, followed by dilution in DMEM with 10% FCS, antibiotics and L-glutamine. Cells were counted, followed by plating in DMEM and culturing in a humidified 5% CO_2 atmosphere at 37ºC.

4.4 Cell proliferation assay

3×10^4 HeLa or Fucci HeLa cells transfected with empty vector mock, c-$FLIP_L$-WT, c-$FLIP_L$-S227A, c-$FLIP_L$-S227D or c-$FLIP_S$ plasmids were plated in 6-well plates, in duplicates, for 4 day-experiments. Every 24 hour, one of the samples was used for cell counting using a hemocytometer, followed by lysis in laemmli buffer (62.5 mM TRIS-HCl pH 6.8, 1 % SDS, 10 % glycerol, 0.005 % bromophenol blue and 1 % beta-mercaptoethanol) and heated for 5 to 10 minutes at 98ºC. Each lysate protein was loaded and resolved by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The other sample was fixed with 50 µL of Fixation/Permeabilization solution (BD Biosciences) for 20 minutes, on ice, and washed with 100 µL of BD Perm/Wash Buffer (BD Biosciences), inside an eppendorf tube. Cells were centrifuged and resuspended in 250 µL of BD Perm/Wash Buffer and run by flow cytometry, on BD LSR II (BD Biosciences). Data analysis was performed using FCS Express 6 Flow Cytometry software (De Novo Software).

4.5 SDS-PAGE and western blotting.

For western blot analysis, cells were lysed in laemmli buffer and heated for 5 to 10 minutes at 98ºC. Each lysate protein was loaded and resolved by SDS-PAGE (10-12%) with a buffer containing 250 mM glycine, 25 mM TRIS-base and 0.1 % SDS. The gels were run for 30 minutes using 80 V, followed by 90 minutes at 120 V. The resolved samples were then transferred to nitrocellulose membrane using wet transfer apparatus for 1 hour with 400 mA. The transfer buffer used contained 192 mM glycine, 24 mM TRIS-base and 20 % methanol. Membranes were blocked with 5% milk in PBS and then incubated with the desired primary antibody (table 1), overnight at 4ºC. On the next day, the membranes were washed three times for 7 minutes, and incubated with the appropriate secondary antibody for 1 hour at room temperature. Then, the membranres were washed three times for 10 minutes. Finally, the membranes were exposed with 1:10 SuperSignal™ West Femto Maximum Sensitivity Substrate : SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFisher Scientific).

Table 4.1 List of diluted primary antibodies and respective secondary antibodies used for WB

Primary Antibody	Company	Secondary Antibody	Company
FLIP (7F10)	Enzo Life Sciences, USA	IgG1	SouthernBiotech
FLAG	Cell Signalling Technology, USA	Rabbit	Promega, USA
HSC70	Enzo Life Sciences, USA	Rat	GE Healthcare, UK
Cyclin E	Cell Signalling Technology, USA	IgG1	SouthernBiotech
p27	Santa Cruz Biotechnology, USA	Rabbit	Promega, USA
PCNA	ChromoTek, Germany	Rat	GE Healthcare, UK

4.6 EdU assay

3×10^4 HeLa cells transfected with empty vector mock, c-FLIP$_L$-WT, c-FLIP$_L$-S227A, c-FLIP$_L$-S227D or c-FLIP$_S$ plasmids were plated in 6-well plates, for a 4 days experiment. Every 48 hours, 10 µM of EdU (Invitrogen) was added to 2 mL of culture medium, and incubated in a humidified 5% CO_2 atmosphere at 37ºC, for 2 hours. Subsequently, the cells were harvested and fixed using 50 µL of Click-iT fixative (Invitrogen), for 15 minutes at room temperature, protected from light. This was followed by addition of 100 µL of Click-iT saponin-based permeabilisation and wash reagent (Invitrogen). Next, 250 µL of Click-iT reaction cocktail (Invitrogen) was added to each sample, followed by 30 minutes incubation at room temperature, protected from light. The samples were washed before staining with 250 µL of FxCycle PI/RNase Staining Solution (Invitrogen), for 15 minutes. Finally, samples were run on BD LSR II and analysed by FCS Express 6 Flow Cytometry software.

4.7 Fucci cell cycle

3×10^4 Fucci HeLa cells transfected with empty vector mock, c-FLIP$_L$-WT, c-FLIP$_L$-S227A or c-FLIP$_L$-S227D plasmids were plated in 6-well plates, for a 2 day-experiment. Every 24 hours, samples were harvested and fixed with 50 µL of Fixation/Permeabilisation solution for 20 minutes, on ice, and washed with 100 µL of BD Perm/Wash Buffer, inside an eppendorf tube. Cells were centrifuged and resuspended in 250 µL of BD Perm/Wash Buffer and run by flow cytometry, on BD LSR II. Data analysis was performed using FCS Express 6 Flow Cytometry software.

4.8 Double thymidine block

3×10^4 Fucci HeLa cells transfected with empty vector mock or c-FLIP$_L$-WT plasmids were plated in 6-well plates. After 12 hours, cells were washed with PBS, DMEM was added with 2 mM of thymidine for 18 hours. To remove first thymidine block, cells were washed with PBS and fresh DMEM was added for 9 hours. Cells were, once again, washed with PBS, and DMEM with 2 mM of thymidine was added for the second thymidine block. After 17 hours, cells were released from block by one wash of PBS and addition of fresh DMEM. Cells were harvested at 0, 2, 4, 6, 10 and 12 hours after second thymidine block release and fixed with 50 µL of Fixation/Permeabilization solution for 20 minutes, on ice, and washed with 100 µL of BD Perm/Wash Buffer, inside an eppendorf tube. Cells were centrifuged and resuspended in 250 µL of BD Perm/Wash Buffer and run by flow cytometry, on BD LSR II. Data analysis was performed using FCS Express 6 Flow Cytometry software.

5 Results

5.1 S227A c-FLIP$_L$ lead to slower cell proliferation

Previous unpublished data from the lab (figure 5.1), shows differences in cell population size upon overexpression of various c-FLIP$_L$ plasmids. This figure shows us transfection of that the c-FLIP plasmid with phosphodeficient mutation (S227A), causes the cells to proliferate less when compared to cells transfected with the empty vector (mock). Additionally, overexpression of c-FLIP$_L$ WT makes the cells grow faster than those transfected with mock. This results suggest that c-FLIP$_L$ has an impact on cell proliferation.

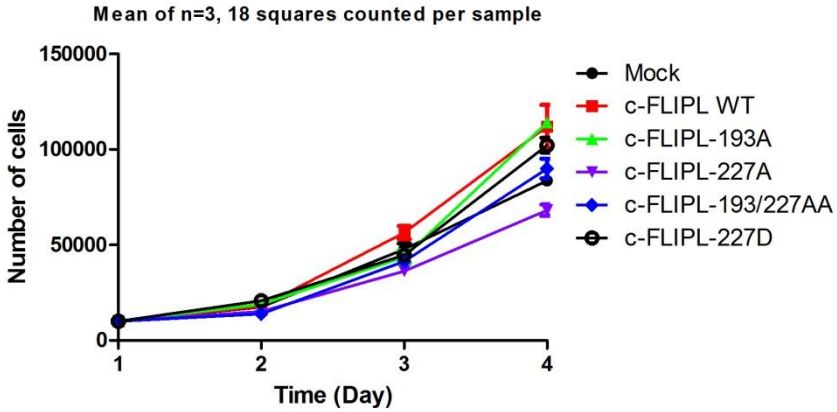

Figure 5.1 Serine to alanine mutation at 227 site cause cells to proliferate slower than WT. HeLa cells were transfected with different plasmids, and counted in a 4 day-experiment. Overexpression of c-FLIP$_L$ 227A and c-FLIP$_l$ 193/227AA lead to slower cell proliferation, while the 193A shows similar number count as WT.

For the continuation of the project, both HeLa and Fucci HeLa cells were used and transfected with mock, WT, S227A and S227D plasmids. The cells were harvested 24h, 48h, 72h and 96h after transfection, and counted with a hemocytometer. After 24 hours of transfection, the number of cells was on average 0.4×10^5 cells (figure 5.2). After four days, it is noticeable that S227A has on average less cells (2.4×10^5 cells) than mock, WT and S227D transfected cells (3.3×10^5 cells). The same results were obtained using Fucci HeLa cells, which started with an average of 0.35×10^5 cells. At the fourth day, S227A cells had a smaller cell population size (2.6×10^5 cells) than mock, WT and S227D (3.9×10^5 cells).

The results obtained from both HeLa and Fucci HeLa cells were similar to the previous experiments from the lab (figure 5.1). However, no significant changes between the cells transfected with mock, WT and S227D plasmids were found.

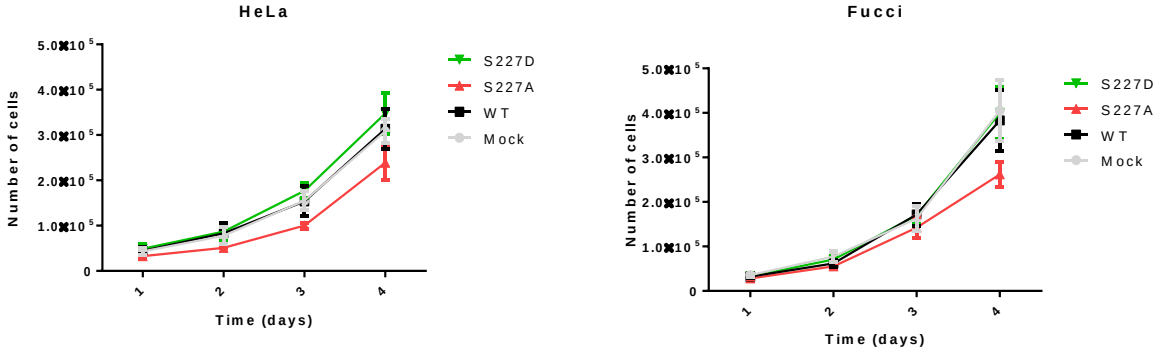

Figure 5.2 Serine to alanine mutation at 227 site causes cells to grow slower than mock, WT and S227D. HeLa and Fucci HeLa cells were transfected with different plasmids, and counted in a 4 days experiment. Overexpression of c-FLIP$_L$ S227A lead to slower cell proliferation when compared to Mock, WT and S227D. Cells were counted using a hemocytometer. Two experimental replicates were made.

To further investigate how c-FLIP$_L$ and its phosphorylation site in S227 affect cell proliferation, the cells were stained with CFSE, followed by transfection with mock, WT, S227A and S227D. Once again, both HeLa and Fucci HeLa cells were used and harvested 24, 48, 72 and 96 hours after transfection, and they were fixed and analysed by flow cytometry. CFSE staining makes cells fluorescent, which will get weaker every time the cells divide. The analysis gives a histogram (figure 5.3), which contains information related how many times cells have divided and the percentage of cells per division. As cells divide, it is expected that the fluorescence of the population decreases, causing a shift to the left.

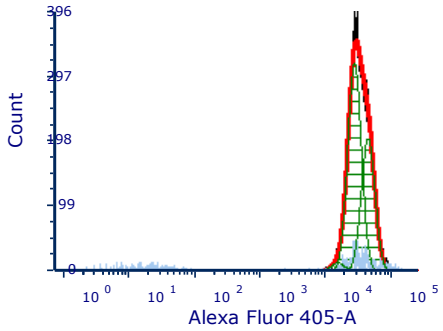

Figure 5.3 Representation of a histogram created from the data obtained by flow cytometry. Alexa fluor 405-A axis represents the intensity of fluorescence detected by the instrument. As the cells divide, the intensity decreases, causing a shift of the population to the left of the axis. Data was obtained from BD LSR II and analysed using FCS Express 6 Plus.

On the first day, most HeLa cells had undergone 2 divisions (figure 5.4). From the data, it is noticeable that the cells transfected with S227A had a higher percentage at the population that divided only once, when compared to the others. From the cells that divided two times, there were also less cells in the S227A-transfected cells compared to mock, WT

and S227D. Therefore, from the first day, it seems that the phosphodeficient S227A mutant of c-FLIP$_L$ causes the cells divide slower. This delayed profile of S227A cells is maintained along the following days. However, no obvious differences were found between the mock, WT and S227D cells (figure 5.4). When experimenting with Fucci HeLa cells, the same profile of cells was found (figure 5.5). Cells transfected with S227A plasmids show also a delayed profile when compared to the other cells, from day one to day four. However, it seems that mock Fucci cells divide faster than the WT and S227D cells, which was not seen in HeLa cells (figure 5.5).

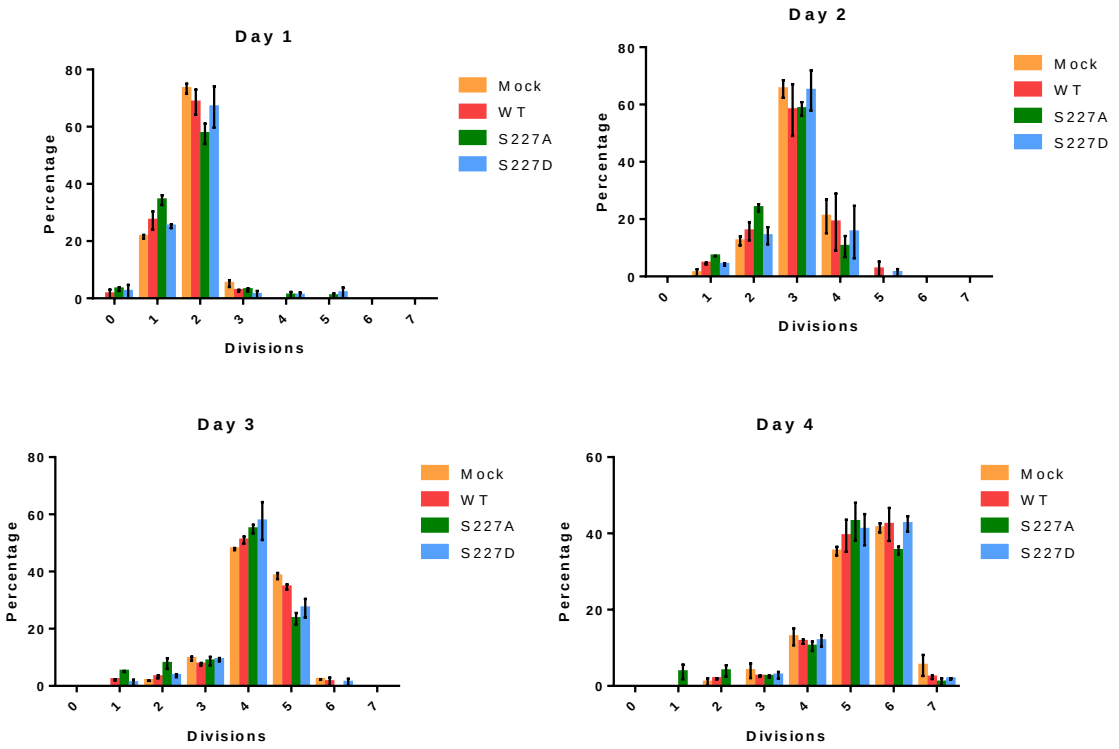

Figure 5.4 S227A cells proliferate slower than mock, WT and D227. HeLa cells were transfected with mock, WT, S227A and S227D to study their effects on proliferation. It was found that S227A proliferates slower than mock, WT and S227D. Days represent time after transfection at which the cells were harvested. Data was obtained from BD LSR II and analysed using FCS Express 6 Plus. Two experimental replicates were made.

5.2 c-FLIP S227A-transfection causes accumulation of cells at G1 phase

From the previous results, it was found that the phosphodeficient c-FLIP$_L$ might affect cell division, causing them to proliferate slower when compared to other transfected cells. Thus, to further investigate this, Fucci cells were used to study cell cycle progression. For this purpose, Fucci cells were transfected with mock, WT, S227A and S227D plasmids, and plated. Cells were then harvested after 24 and 48 hours after transfections, fixed and analysed

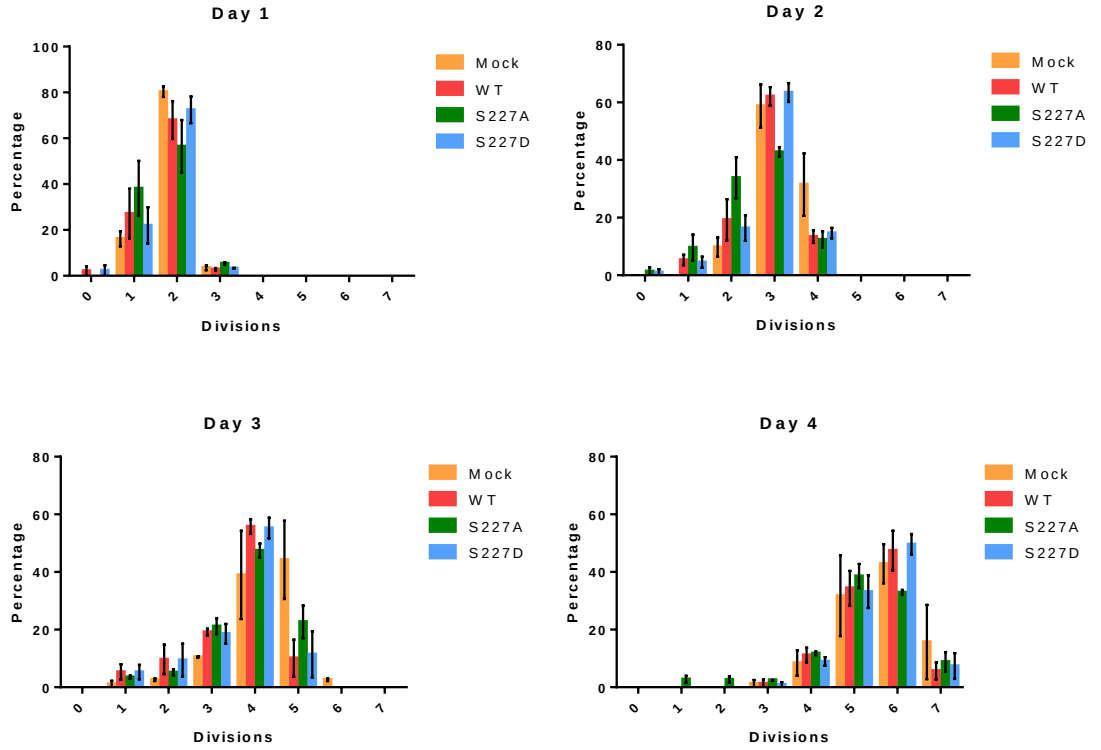

Figure 5.5 S227A cells are slower proliferating than mock, WT and D227. Fucci HeLa cells were transfected with mock, WT, S227A and D227 to study their effects on proliferation. It was found that S227A proliferates slower than mock, WT and D227. Furthermore, it seems that mock cells are growing faster than WT and D227. Days represent time after transfection at which the cells were harvested. Data was obtained from BD™ LSR II and analysed using FCS Express 6 Plus. Two experimental replicates were made.

using flow cytometry. Fucci technology allows easy determination of the different cell cycle phases using constructs that give orange colour to G1 phase nuclei, and green colour to S/G2/M phases nuclei. At an early stage of G1 phase, the cells are not labelled. When analysing Fucci HeLa cells with flow cytometry, a dot plot (figure 5.6) can be obtained. The cells located in the lower left quadrant are those which do not have fluorescence, corresponding at an early G1 phase. The G1 phase is located at the top left quadrant, while G2/M phase is at the lower right side of the dot plot. Lastly, the top right quadrant represents cells which are labelled with green and orange colour simultaneously, which were considered to be in S phase (figure 5.6).

From figure 5.7, it is evident that after 24 hours of transfection, mock-transfected cells have most cells committed to division, with 40.56% of cells at the S phase and 20.04% at G2/M phase. On the second day, most mock-transfected cells are preparing for mitosis, with 7.99% at an early G1 phase and 40.62% at G1 phase. Similarly, most cells transfected with WT plasmid are committing to divide, with 39.47% of cells at S phase and 17.79% at G2/M.

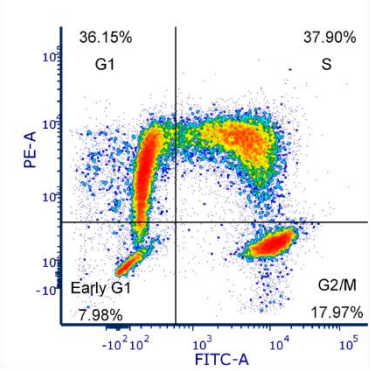

Figure 5.6 Representation of a dot plot obtained from Fucci HeLa cells for cell cycle studies. FITC-A axis represents the intensity of green fluorescent detected, while PE-A axis represents intensity of the orange colour. The percentages of cells per quadrant are shown. The lower left quadrant represents early G1 phase cells, while the top left cells in G1 phase. The top right quadrant contains cells in S phase, while lower right contains cells from G2/M phase. Data was obtained from BD LSR Fortessa and analysed using FCS Express 6 Plus.

On the second day, these WT cells, also have a cell cycle profile similar to the mock cells. However, some differences were found with S227D cells, where there was a lower percentage of cells at G2/M phase (17.05%) on day 1 when compared to mock and WT. However, the percentage of S227D cells at early G1 and G1 phase was 45.75%, which was higher than in mock (39.41%) and WT (42.74%). This difference is still noticeable on the second day, where S227D cells have a higher percentage of cells prepared to divide, when compared with both mock and WT. This suggests that cells overexpressing c-FLIP$_L$ with a phosphomimicking mutation could be dividing faster. Moreover, S227A cells show an increased population in an early G1 phase at day 1 (13.84%), while other transfected cells had between 5 to 8% of cells in that phase. This accumulation of G1 population for S227A cells remained on the second day, having reached 20.90% of cells in early G1 phase, while other cells reached only 8 to 12%. Additionally, it seems that the S227A mutation slows entry into the S phase, since there were only 26.73% of cells in this 17.95%phase, while mock, WT and S227D had more than 30% of cells in S-phase.

Similar results could be obtained using HeLa cells. However, these cells need to be treated with EdU for 2 hours, followed by harvesting and Click-iT reaction. Further, the cells were stained with FxCycle™ PI/RNase staining solution, just before being analysed by flow cytometry. However, when this staining was performed in the transfected samples, we did not obtain the expected profile when analysing by flow cytometry. When the staining works as it should, the profile should be similar to the figure 5.8A. From that profile, we can obtain the percentage of cells in G1, S and G2 phase, because cells have different fluorescent

intensities of EdU and PI dependent on the cell cycle phase. However, when we tried the staining using our transfected samples, we obtained the profile shown figure 5.8B, which was not expected and does not allow us to analyse the cell cycle of HeLa cells.

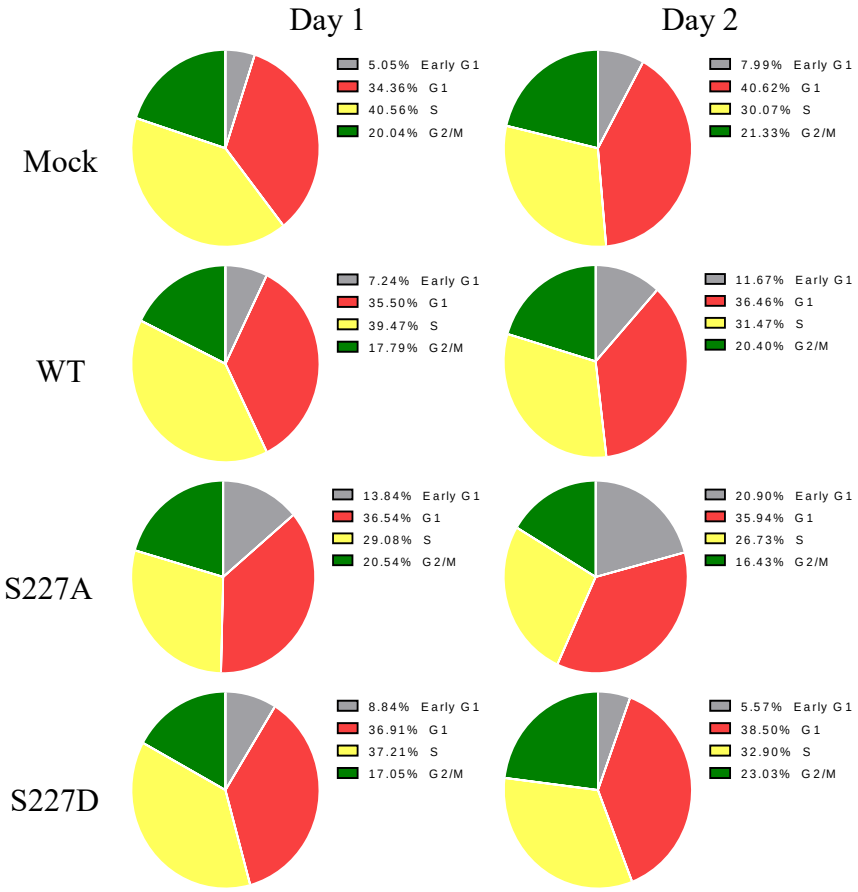

Figure 5.7 S227A shows an increased population at an early G1 and G1 phase when compared to mock, WT and S227D. Fucci HeLa cells were transfected with mock, WT, S227A and S227D to study their effects on cell cycle progression. S227A has an accumulating population at early G1 and G1 phases. S227D seems to be progressing through the cell cycle faster than mock and WT. Days represent the time after transfection that the cells were harvested. Data was obtained from BD LSR Fortessa and analysed using FCS Express 6 Plus. Two experimental replicates were made.

5.3 c-FLIP WT-transfected cells seem to progress through cell cycle faster than mock

Although some differences between the transfected cells were found with the previous experiment, it was not sufficient to pinpoint if the cells overexpressing c-FLIP WT and mutants were faster or slower to divide than the mock cells. Thus, an experiment where Fucci HeLa cells transfected with either mock or WT plasmids were synchronised, was performed. These cells were synchronised using double thymidine block, which blocked the cells

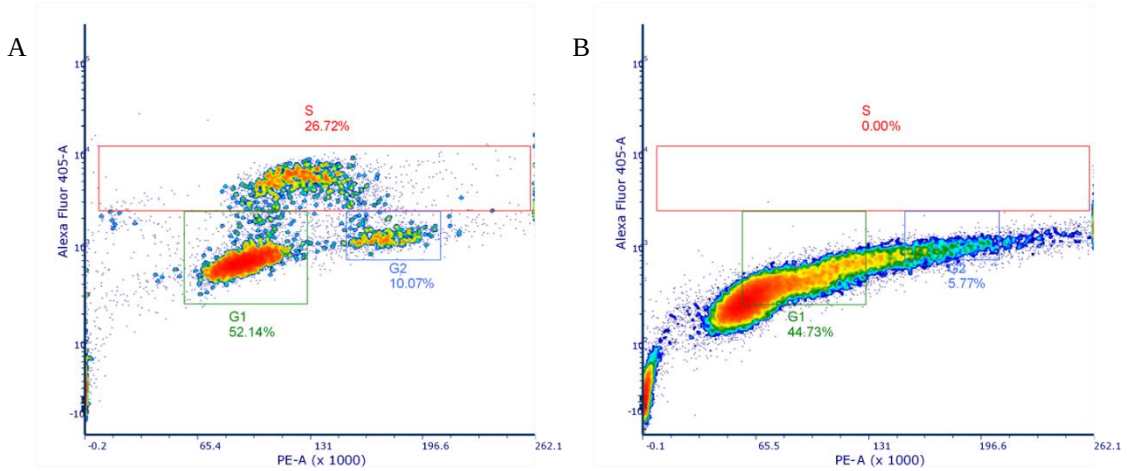

Figure 5.8 Representation of a dot plot obtained from HeLa cells for cell cycle studies. PE-A axis represents the intensity of PI, while Alexa fluor 405-A axis represents intensity of fluorescence obtained from Click-iT reaction. The percentages of cells per region are shown, as well as which cell cycle phase does each region represent. Data was obtained from BD LSR II and analysed using FCS Express 6 Plus.

throughout the S phase, and they were harvested 0, 2, 4, 6, 10 and 12 hours after being released. The cells were then fixed and analysed by flow cytometry. Cells that were not synchronised were used as control of the synchronisation method. Figure 5.9 shows that thymidine block worked, since the profile of unsynchronised and synchronised cells is different. However, the first two time points did not show the expected profile, since thymidine block arrest cells at the G1/S phase, and these samples show a significant percentage of cells at G2/M (figure 5.9). After 4 hours, the obtained profiles start to be as expected, because most cells are in G2/M phase. At 6 hours after release, there was an increase of 9.57% in mock and 6.97% in WT, in G2/M population. Differences in the cell cycle profile started to be noticeable at the 10th hour after release, when most of the cells reach the G1 phase. Mock cells, had an increase of 19.55% and 39.33% in early G1 and G1 populations, respectively. WT, in their turn, had an increase of 14.3% in early G1 phase, while G1 phase the increase reached 41%. This suggests a difference of cell cycle progression between WT and mock, which is bigger after 12 hours. When we compared the population in early G1 phase, it was found that WT cells had only 14.60%, a lower percentage than mock (24.86%). However, when we looked at the G1 phase, the percentages of WT were higher than mock 7.94%. This could suggest that WT cells are dividing faster than mock.

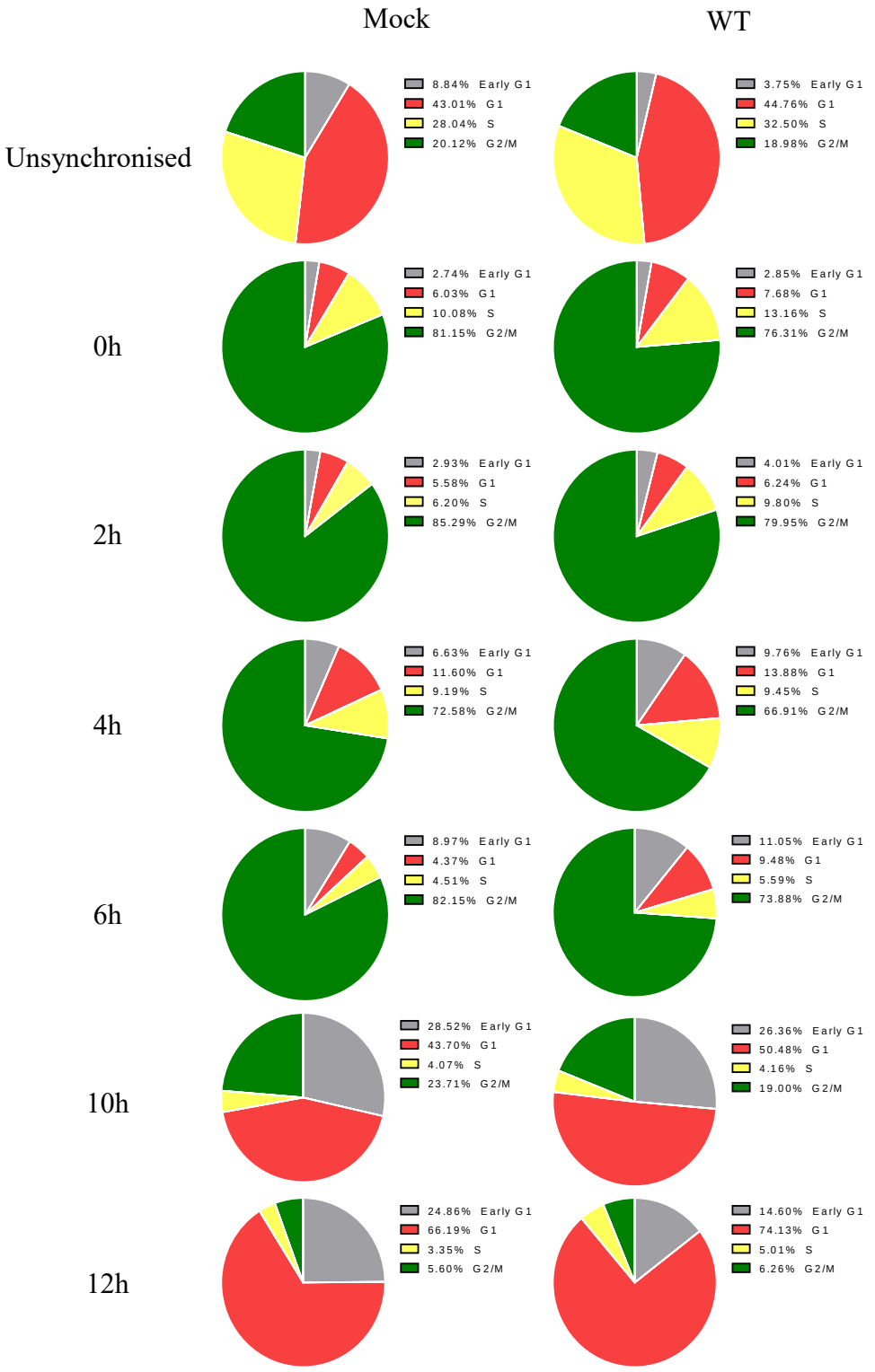

Figure 5.9 WT progress faster through cell cycle. Fucci HeLa cells were transfected with mock and WT, followed by thymidine block, to study which transfected population was faster progressing through the cell cycle. WT seems to be progressing through the cell cycle faster than mock. Hours represent the time which the cells were harvested after being released from thymidine block. Data was obtained from BD LSR Fortessa and analysed using FCS Express 6 Plus. One experimental replicate was made.

5.4 S227A-transfected cells have decreased levels of cyclin E

It was previously referred that c-FLIP$_L$ overexpression could influence the cell cycle, more specifically at the G1/S transition. Thus, from the cells plated for CFSE experiments, one of the duplicates was collected for western blotting to study how cell cycle regulators levels are affected by mock, WT, S227A and S227D transfections, along the four days of experiments. First, we blotted for c-FLIP$_L$ to study transfection efficiency. From the blots, three bands were found: the lower one represents p43-FLIP, which is a cleaved form of c-FLIP$_{L;}$ the middle band is c-FLIP$_L$, while the top is the FLAG-tagged c-FLIP$_L$, produced from the plasmid that was transfected (figure 5.10). The blots confirmed that there was overexpression of c-FLIP$_L$ throughout the four days, although S227A cells had less amount of c-FLIP$_L$ than WT and S227D. Cyclin E, which is a major regulator of G1/S transition, was also studied. From the first day, it seems that the phosphomimicking mutation stimulates cyclin E expression, while the phosphodeficient mutation causes a decrease of it. On the second day, it was found that both WT and S227D lead to more of cyclin E when compared to mock and S227A. This was also found on the third day, although the mock sample showed more cyclin E than the others. This could explain why cells overexpressing WT and S227D grow faster than S227A cells (figure 5.10). p27, which is another important regulator of G1/S transition, was also studied. Differences were only found on the first and fourth day after transfection. After 24 hours, it was found that WT and S227D cells had more p27 than the others, while on the fourth day this was found in both mock and S227D. Both results are not as expected, since overexpression of c-FLIP$_L$ leads to p27 depletion. Proliferating cell nuclear antigen (PCNA), an important component of the nucleic acid metabolism and cell cycle regulation, and used as proliferative marker, was also studied. From the first and second day, it was found that S227A cells had weaker bands when compared to the other transfected cells. No differences were found on the third day, and on the last day it seems that the WT transfected cells had less amount of PCNA. Heat shock 70 kDa protein 8 (Hsc70) was the protein used as a loading control. No significant differences on the loading were found, except for the fourth day, which seemed to have had less amount of S227D loaded, which could be due to bad blotting.

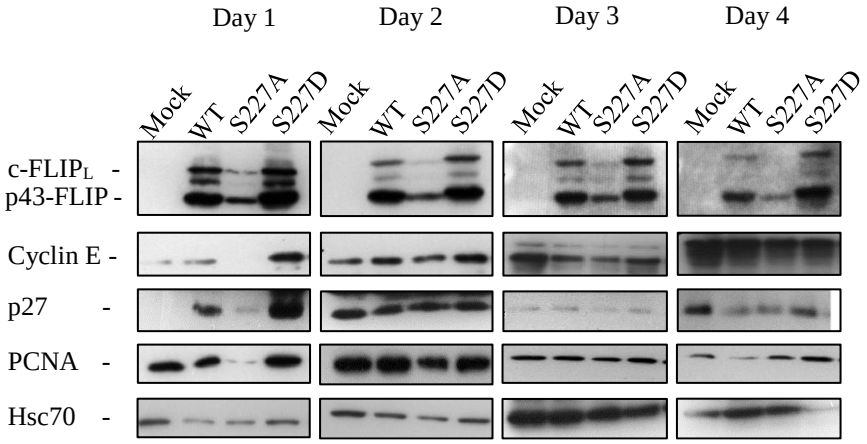

Figure 5.10 Cyclin E levels are lower in S227A. c-FLIP$_L$ was shown to be overexpressed in WT, S227A and S227D samples. Cyclin E shows low levels in S227A, and higher levels in D227. p27 and PCNA blots do not show a consistent profile across the different days. Hsc70 was used as a loading control. HeLa cells were transfected with mock, WT, S227A and S227D. After, they were harvested at different time points after transfection, run in a 10-12 % SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated with the desired primary and secondary antibodies, followed by development with enhanced chemiluminescence (ECL) western blotting substrate. Two experimental replicates were made.

Fucci HeLa cells samples were also used for western blotting. Once again, when blotted with anti-c-FLIP antibody it was found that there was overexpression of WT, S227A and S227D (figure 5.11). However, mock samples from the second, third and fourth day showed to have c-FLIP expression comparable to the overexpressed samples, which should not occur, because mock is an empty vector that cannot overexpress c-FLIP$_L$. While studying cyclin E, it was found that, on the first day, S227D had stronger band when compared to other, with mock sample having the weaker band. On the following day, the WT sample had the weaker band, while on the fourth day no significant differences were found, although mock transfected cells appear to have a stronger band than others (figure 5.11). With PCNA, it was found that, on the first and second day, S227D sample had a stronger band than the others, although on the second day S227A had a band with similar intensity than S227D. However, on the fourth day, S227A showed a much weaker band than the others, which would suggest less proliferation of these cells. Again, Hsc70 was used as a loading control, which showed differences in the loading of the third and fourth day, which higher amount of mock and WT samples.

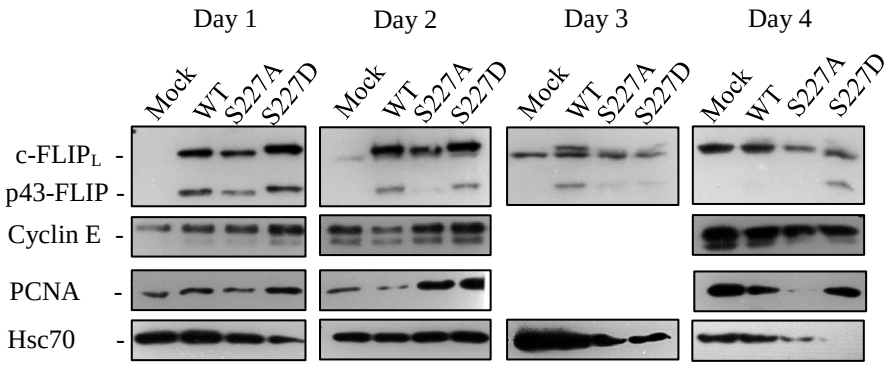

Figure 5.11 c-FLIP$_L$ was shown to be overexpressed in WT, S227A and S227D samples. Cyclin E shows high levels in S227D, however other samples do not show consistent band intensity upon different days. PCNA blots also do not show a consistent profile across the different days. Hsc70 was used as a loading control. Fucci HeLa cells were transfected with mock, WT, S227A and S227D. After, they were harvested at different time points after transfection, run in a 10-12 % SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated with the desired primary and secondary antibodies, followed by development with ECL western blotting substrate. Two experimental replicates were made.

6 Discussion

Since the discovery of c-FLIP, most studies were concerning the anti-apoptotic roles of this protein. These roles were found to be more related to the short form of c-FLIP, while c-$FLIP_L$ could also have pro-apoptotic roles. Furthermore, some reports started to show evidence that the long form of this protein could have roles in cell proliferation and cell-cycle progression (107). Moreover, among the different ways to control c-FLIP, previous unpublished work from our laboratory suggested that phosphorylation at serine 227 could have an effect in cell proliferation, while c-$FLIP_L$ was being overexpressed. Thus, we started by studying cell population while overexpressing c-$FLIP_L$ WT, S227A or S227D. These results showed, for both HeLa and Fucci HeLa cells, that S227A cells had fewer cells after four days of experiment, when compared to mock, WT and S227D. This suggests that the serine to alanine mutation, which cannot be phosphorylated due to lack of a hydroxyl group, affects the cells capacity to proliferate as fast as the others. However, the number of cells obtained from the mock transfected cells were higher than expected, since unpublished data from our lab had shown that mock cells proliferate slower than WT and S227D. Additionally, we studied the proliferative behaviour of cells through the CFSE method, which allowed us to understand how fast cells were proliferating (115). We found that cells overexpressing phosphodeficient c-$FLIP_L$ proliferate slower, when compared to the other cells. Overall, a bigger population of S227A cells was found to undergo less one division than mock, WT and S227D. Once again, during these experiments we saw the mock population to grow at the same rate as WT and S227D, or even faster, in the case of Fucci HeLa cells. This could occur because of problems with the plasmid used for transfection, which might be stimulating some cellular mechanism, causing cell to proliferate faster than expected. Based on previous experiments, we expected both WT and S227D to be the fastest growing cells, and S227A to be the slowest ones. These results confirm S227A mutation affects cell proliferation. Moreover, this slower proliferation profile found through CFSE method could explain why cell counts were smaller in the previous experiment.

Western blot was also performed to study the expression of PCNA, a proliferative marker. Weaker bands in S227A samples from HeLa cells were found, which was expected, since this cells have been shown to be the ones with slower proliferation. Further, in samples from Fucci HeLa cells, we found stronger bands of PCNA in S227D samples, which was expected due to faster proliferation. However, mock and WT showed weaker bands than S227D, even

though in cell counting experiments and CFSE method they showed to behave similarly to S227D cells. Additionally, the results obtained from PCNA blots, were not entirely consistent with the observations made using proliferative methods.

The G1/S transition of the cell cycle is crucial for the cells, since an "all-or-none switch" is created, which forces the cells to divide, only stopping if errors occur along the division mechanism (121). Thus, this transition can control the rate the cells are proliferating at. To further understand why these S227A transfected cells grow slower than the others, we studied the cell cycle profile of these cells. We stained HeLa cells with EdU and PI, however, the results were not as expected, since we could not differentiate proliferating cells from the non-proliferating. From these results, it would seem that either the cells did not incorporate EdU, or they incorporated it, but the Click-iT® reaction was not successful. Nevertheless, using HeLa cells stably expressing Fucci, we were able to follow cell division. Additionally, using flow cytometry analysis, it was possible to calculate the percentage of cells within each cell cycle phase (122,123). From this, we found that cells overexpressing phosphodeficient c-FLIP$_L$ had much higher percentage of cells at early G1 phase, when compared to the other transfected cells. These differences were even bigger on second day after the transfection, which suggests that the mutation is affecting the normal progression of cell cycle and, consequently, making them proliferate slower. Overexpression of c-FLIP$_L$ caused a decrease of p27 protein levels, which is a known CKI that inhibits cyclin E-CDK2 (106). Moreover, p27 protein was suggested to inhibit G1/S transition (27). Thus, S227A cells might be accumulated at an early G1 phase, because the phosphodeficient mutation makes c-FLIP$_L$ unable to stimulate a p27 decrease, and so the cells do not progress to S phase as easily. Additionally, we found a small difference between S227D, WT and mock cells, which suggests that S227D cells were progressing faster throughout the cell cycle. However, when looking at the levels of p27 via western blot in HeLa cells, the differences found were not the expected, since WT and S227D had higher levels of p27 when compared to mock and S227A. Since cyclin E is one of the targets of p27 protein, we searched if its levels were affected in WT and S227D cells. From HeLa cells samples, we found evidence that cyclin E can be stimulated by c-FLIP$_L$ S227D and WT overexpression, even though the intensity of p27 bands were either stronger or similar to mock samples. This could suggest that the levels of cyclin E were enough to overcome p27 inhibition, without the need of decreasing p27 levels. It was shown that cyclin E overexpression could shorten G1 phase, by premature

activation of cyclin E-CDK2 complex (9). Thus, it is possible that these higher levels of cyclin E found in S227D and WT samples are the reason why these cells progress faster to S phase, making them grow faster than S227A transfected cells.

Although using unsynchronised Fucci HeLa cells to study cell cycle profile showed us that there was an accumulating number of cells at early G1 phase in S227A samples, differences between the other samples were hard to identify. Therefore, a preliminary experiment using synchronised Fucci HeLa cells was also performed. This was done to verify if we could overcome the problem of understanding if one cell population was being faster or slower than the other. This way, we could analyse how the cells progress through the cell cycle phases, while starting from the same phase. Samples from 0 and 2 hours after release showed a high percentage of cells at G2/M, which is not correct because double thymidine block arrest cells at G1/S transition. It is possible that one of the two blocking step was not successful, allowing cells to progress until G2/M phase. However, after 4 hours the profile starts to be as expected (124), followed by an increase of G2/M population at 6 hours after release. This preliminary result showed at 10 hours a small difference between the WT and mock, in which WT had a higher percentage of cells at G1 phase, which was even more noticeable at 12 hours after thymidine release. This could suggest that WT cells were dividing faster than the mock cells. Nevertheless, further trials need to be done, including samples of cells overexpressing c-FLIP$_L$ mutants.

From the data we collected, it would seem that c-FLIP$_L$ has indeed a role in cell proliferation while being overexpressed. Furthermore, we found that throughout the four days of experiments, cyclin E levels of WT and S227D cells were higher than S227A cells, even though a decrease in p27 levels was not found. This can suggest that c-FLIP$_L$ overexpression stimulates G1/S transition without changing p27 protein levels. We found that, while overexpressing c-FLIP$_L$, a big amount of this protein was cleaved into p43-FLIP fragment. Reports have shown that c-FLIP$_L$ could interact with NF-κB by this fragment (90,101). Additionally, p22-FLIP fragment was found to be interacting and activating IKK complex (92,102). Also, studies have shown that inactivating NF-κB can cause delay in G1/S transition (125). Thus, it could also be possible that the overexpression will cause an increase in interaction of c-FLIP$_L$ fragments with NF-κB and IKK complex, which would lead to faster G1/S transition and, subsequently, increased cell proliferation. However, studying c-

$FLIP_L$ cleavage into p22-FLIP and p43-FLIP, as well as interaction studies with NF-κB and IIK complex are needed to further investigate this.

How c-$FLIP_L$ overexpression affects the cell population can be important for tumour growth. It was shown that this protein is frequently upregulated in some human tumours (126,127). Further, it was suggested that it could affect cell cycle progression and increase cell proliferation in carcinogenesis (107,128). The data we collected is in agreement with these suggestions, showing that overexpression of c-$FLIP_L$ affects cell proliferation and cell cycle progression. Further, our data suggests that this is controlled by S227 phosphorylation. Due to its anti-apoptotic role, c-FLIP has also been studied as a target for cancer therapy, with different approaches being tested (129–131). Studies have shown that targeting c-FLIP could represent an effective anti-tumour therapy. Our findings can further support this claim, since targeting c-$FLIP_L$ can also reduce cell proliferation.

7 Concluding remarks and future perspectives

c-FLIP$_L$ was first identified as having only an anti-apoptotic role, though now it is known that it can control cell fate by affecting different cell mechanisms. Some groups have suggested that c-FLIP$_L$ can have a role in controlling cell proliferation and cell cycle, even though the exact mechanism by which c-FLIP controls these processes is still unknown. Our study shows that overexpression of c-FLIP$_L$ can indeed affect cell proliferation, and indicate that phosphorylation at S227 is important for it. Further, our data regarding the cell cycle showed that the phosphodeficient mutation at 227 lead to accumulation of cells across G1 phase. Nonetheless, further repetitions of these experiments need to be performed due to unexpected results obtained from the mock transfected cells. After these repeats, we might be able to infer if overexpression of WT c-FLIP$_L$ grows indeed faster than the control cells, and which are the differences found at the cell cycle progression. Furthermore, synchronising cells need to be performed in order to further study cell cycle progression. From this, we can infer about the time one population needs to complete the cell cycle. We will also repeat the EdU protocol, which will give us further information about the cell cycle progression in HeLa cells. The blots will also be repeated due to inconsistent results that we have obtained during this experiments. Cells expressing fluorescent-tagged cyclin E, fluorescent-tagged cyclin A or fluorescent-tagged p27 proteins transfected with mock, WT, S227A and S227D will be used and analysed using flow cytometry. From these results, since the fluorescent is proportional to the levels of protein in the cell, we can search for differences in protein levels in the different transfected cells. We are also planning on understanding which is the enzyme responsible for phosphorylating c-FLIP$_L$ at S227. Chick chorioallantoic membrane assay might also be used as an *in vivo* model to study the effects of c-FLIP$_L$ overexpression on cell population size.

c-FLIP$_L$ upregulation in some human tumours, added to the fact that it affects cell proliferation while overexpressed, makes it a promising target for cancer therapy. However, its resemblance to caspase-8 makes c-FLIP$_L$ targeting a bigger challenge. Thus, further studies need to be done in order to target c-FLIP$_L$ without affecting caspase-8 levels. Therefore, further study S227 phosphorylation as the regulator of c-FLIP$_L$ proliferative role could help us understand if this phosphorylation could be a possible target for cancer therapy.

8 References

1. Alberts B. Molecular biology of the cell. Sixth edition. New York: Garland Science, Taylor and Francis Group; 2015
2. Pardee AB, Dubrow R, Hamlin JL, Kletzien RF. Animal cell cycle. Annu Rev Biochem. 1978;47(1):715–750.
3. Iftode C, Daniely Y, Borowiec JA. Replication Protein A (RPA): The Eukaryotic SSB. Crit Rev Biochem Mol Biol. 1999 Jan;34(3):141–80.
4. Elledge SJ. Cell cycle checkpoints: preventing an identity crisis. Science. 1996;274(5293):1664.
5. Morgan DO. Principles of CDK regulation. Nature. 1995;374(6518):131–4.
6. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. 2003;36(3):131–149.
7. Evans T, Rosenthal ET, Youngblom J, Distel D, Hunt T. Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell. 1983;33(2):389–96.
8. Assoian R, Zhu X. Cell anchorage and the cytoskeleton as partners in growth factor dependent cell cycle progression. Curr Opin Cell Biol. 1997;9(1):93–8.
9. Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol Cell Biol. 1995;15(5):2612–2624.
10. Girard F, Strausfeld U, Fernandez A, Lamb NJ. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell. 1991;67(6):1169–1179.
11. King RW, Jackson PK, Kirschner MW. Mitosis in transition. Cell. 1994;79(4):563–71.
12. Glotzer M, Murray AW, Kirschner MW. Cyclin is degraded by the ubiquitin pathway. Nature. 1991;349(6305):132–8.
13. De Bondt HL, Rosenblatt J, Jancarik J, Jones HD, Morgant DO, Kim S-H. Crystal structure of cyclin-dependent kinase 2. Nature. 1993;363(6430):595–602.
14. Fesquet D, Labbe JC, Derancourt J, Capony JP, Galas S, Girard F, Lorca T, Shuttleworth J, Dorée M, Cavadore JC. The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. EMBO J. 1993;12(8):3111.
15. Lew DJ, Kornbluth S. Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. Curr Opin Cell Biol. 1996;8(6):795–804.
16. Carnero A, Hannon GJ. The INK4 family of CDK inhibitors. Curr Top Microbiol Immunol. 1998;227:43–55.
17. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 1999 Jun 15;13(12):1501–12.
18. Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. 1994;8(1):9–22.
19. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature. 1993 Dec;366(6456):701–4.
20. Pardee AB. G1 events and regulation of cell proliferation. Science. 1989 Nov 3;246(4930):603–8.

21. Terada Y, Inoshita S, Nakashima O, Kuwahara M, Sasaki S, Marumo F. Regulation of cyclin D1 expression and cell cycle progression by mitogen-activated protein kinase cascade. Kidney Int. 1999 Oct;56(4):1258–61.

22. Giacinti C, Giordano A. RB and cell cycle progression. Oncogene. 2006;25(38):5220–7.

23. Bähler J. Cell-cycle control of gene expression in budding and fission yeast. Annu Rev Genet. 2005;39:69–94.

24. Henley SA, Dick FA. The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. Cell Div. 2012;7(1):10.

25. Cobrinik D. Pocket proteins and cell cycle control. Oncogene. 2005 Apr 18;24(17):2796–809.

26. Skotheim JM, Di Talia S, Siggia ED, Cross FR. Positive feedback of G1 cyclins ensures coherent cell cycle entry. Nature. 2008 Jul 17;454(7202):291–6.

27. Barr AR, Heldt FS, Zhang T, Bakal C, Novák B. A Dynamical Framework for the All-or-None G1/S Transition. Cell Syst. 2016 Jan;2(1):27–37.

28. Xu M, Sheppard K-A, Peng C-Y, Yee AS, Piwnica-Worms H. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol Cell Biol. 1994;14(12):8420–8431.

29. DeGregori J, Kowalik T, Nevins JR. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Mol Cell Biol. 1995 Aug;15(8):4215–24.

30. Montagnoli A, Fiore F, Eytan E, Carrano AC, Draetta GF, Hershko A, Pagano M. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev. 1999;13(9):1181–1189.

31. Morkel M, Wenkel J, Bannister AJ, Kouzarides T, Hagemeier C. An E2F-like repressor of transcription. Nature. 1997 Dec 11;390(6660):567–8.

32. Westendorp B, Mokry M, Groot Koerkamp MJA, Holstege FCP, Cuppen E, de Bruin A. E2F7 represses a network of oscillating cell cycle genes to control S-phase progression. Nucleic Acids Res. 2012 Apr;40(8):3511–23.

33. Bertoli C, Skotheim JM, de Bruin RAM. Control of cell cycle transcription during G1 and S phases. Nat Rev Mol Cell Biol. 2013 Jul 23;14(8):518–28.

34. Hagting A, Karlsson C, Clute P, Jackman M, Pines J. MPF localization is controlled by nuclear export. EMBO J. 1998 Jul 15;17(14):4127–38.

35. Moore JD, Yang J, Truant R, Kornbluth S. Nuclear Import of Cdk/Cyclin Complexes: Identification of Distinct Mechanisms for Import of Cdk2/Cyclin E and Cdc2/Cyclin B1. J Cell Biol. 1999 Jan 25;144(2):213–24.

36. Parker L, Piwnica-Worms H. Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. Science. 1992 Sep 25;257(5078):1955–7.

37. Draetta G, Eckstein J. Cdc25 protein phosphatases in cell proliferation. Biochim Biophys Acta. 1997 Apr;1332(2):M53-63.

38. Lolli G, Johnson LN. CAK-Cyclin-dependent Activating Kinase: a key kinase in cell cycle control and a target for drugs? Cell Cycle Georget Tex. 2005 Apr;4(4):572–7.

39. Weinert TA, Hartwell LH. The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science. 1988 Jul 15;241(4863):317–22.

40. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature. 2004;432(7015):316–323.

41. Walworth N, Davey S, Beach D. Fission yeast chk1 protein kinase links the rad checkpoint pathway to cdc2. Nature. 1993 May 27;363(6427):368–71.

42. Murakami H, Okayama H. A kinase from fission yeast responsible for blocking mitosis in S phase. Nature. 1995 Apr 27;374(6525):817–9.

43. Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, Elledge SJ. Conservation of the Chk1 Checkpoint Pathway in Mammals: Linkage of DNA Damage to Cdk Regulation Through Cdc25. Science. 1997;277(5331):1497–501.

44. Furnari B, Rhind N, Russell P. Cdc25 Mitotic Inducer Targeted by Chk1 DNA Damage Checkpoint Kinase. Science. 1997 Sep 5;277(5331):1495–7.

45. Peng C-Y, Graves PR, Thoma RS, Wu Z, Shaw AS, Piwnica-Worms H. Mitotic and G2 Checkpoint Control: Regulation of 14-3-3 Protein Binding by Phosphorylation of Cdc25C on Serine-216. Science. 1997 Sep 5;277(5331):1501–5.

46. Baber-Furnari BA, Rhind N, Boddy MN, Shanahan P, Lopez-Girona A, Russell P. Regulation of Mitotic Inhibitor Mik1 Helps to Enforce the DNA Damage Checkpoint. Mol Biol Cell. 2000 Jan 1;11(1):1–11.

47. Appella E, Anderson CW. Post-translational modifications and activation of p53 by genotoxic stresses: p53 post-translational modifications. Eur J Biochem. 2001 May 15;268(10):2764–72.

48. Momand J, Wu HH, Dasgupta G. MDM2 — master regulator of the p53 tumor suppressor protein. Gene. 2000 Jan 25;242(1–2):15–29.

49. Chehab NH, Malikzay A, Stavridi ES, Halazonetis TD. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc Natl Acad Sci. 1999 Nov 23;96(24):13777–82.

50. Shieh S-Y, Ikeda M, Taya Y, Prives C. DNA Damage-Induced Phosphorylation of p53 Alleviates Inhibition by MDM2. Cell. 1997 Oct 31;91(3):325–34.

51. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 1991 Dec 1;51(23 Pt 1):6304–11.

52. Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, Vogelstein B, Jacks T. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev. 1995;9(8):935–944.

53. Stewart ZA, Leach SD, Pietenpol JA. p21(Waf1/Cip1) inhibition of cyclin E/Cdk2 activity prevents endoreduplication after mitotic spindle disruption. Mol Cell Biol. 1999 Jan;19(1):205–15.

54. Bertoli C, Klier S, McGowan C, Wittenberg C, de Bruin RAM. Chk1 Inhibits E2F6 Repressor Function in Response to Replication Stress to Maintain Cell-Cycle Transcription. Curr Biol. 2013 Sep;23(17):1629–37.

55. Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai LH, Zhang P, Dobrowolski S, Bai C, Connell-Crowley L, Swindell E, Fox MP, Wei N. Inhibition of cyclin-dependent kinases by p21. Mol Biol Cell. 1995 Apr 1;6(4):387–400.

56. Jin S, Antinore MJ, Lung FD, Dong X, Zhao H, Fan F, Colchagie AB, Blanck P, Roller PP, Fornace AJ Jr, Zhan Q. The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. J Biol Chem. 2000 Jun 2;275(22):16602–8.

57. Innocente SA, Abrahamson JLA, Cogswell JP, Lee JM. p53 regulates a G2 checkpoint through cyclin B1. Proc Natl Acad Sci U S A. 1999 Mar 2;96(5):2147–52.

58. Taylor WR, DePrimo SE, Agarwal A, Agarwal ML, Schönthal AH, Katula KS, Stark GR. Mechanisms of G2 Arrest in Response to Overexpression of p53. Mol Biol Cell. 1999 Nov 1;10(11):3607–22.

59. Taylor WR, Stark GR. Regulation of the G2/M transition by p53. Oncogene. 2001 Apr 5;20(15):1803–15.

60. Flatt PM, Tang LJ, Scatena CD, Szak ST, Pietenpol JA. p53 Regulation of G2 Checkpoint Is Retinoblastoma Protein Dependent. Mol Cell Biol. 2000 Jun;20(12):4210–23.

61. Jackson MW, Agarwal MK, Yang J, Bruss P, Uchiumi T, Agarwal ML, Stark GR, Taylor WR. p130/p107/p105Rb-dependent transcriptional repression during DNA-damage-induced cell-cycle exit at G2. J Cell Sci. 2005 May 1;118(Pt 9):1821–32.

62. Bertin J, Armstrong RC, Ottilie S, Martin DA, Wang Y, Banks S, Wang GH, Senkevich TG, Alnemri ES, Moss B, Lenardo MJ, Tomaselli KJ, Cohen JI. Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. Proc Natl Acad Sci U S A. 1997 Feb 18;94(4):1172–6.

63. Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schröter M, Scaffidi C, Krammer PH, Peter ME, Tschopp J. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature. 1997;386(6624):517–21.

64. Thome M, Tschopp J. Regulation of lymphocyte proliferation and death by FLIP. Nat Rev Immunol. 2001;1(1):50–58.

65. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schröter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. Inhibition of death receptor signals by cellular FLIP. Nature. 1997;388(6638):190–5.

66. Djerbi M, Darreh-Shori T, Zhivotovsky B, Grandien A. Characterization of the Human FLICE-Inhibitory Protein Locus and Comparison of the Anti-Apoptotic Activity of Four Different FLIP Isoforms. Scand J Immunol. 2001;54(1–2):180–189.

67. Tschopp J, Irmler M, Thome M. Inhibition of fas death signals by FLIPs. Curr Opin Immunol. 1998;10(5):552–558.

68. Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. Nat Rev Immunol. 2007 Jul;7(7):532–42.

69. Chang DW. c-FLIPL is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. EMBO J. 2002 Jul 15;21(14):3704–14.

70. Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J. NF- B Signals Induce the Expression of c-FLIP. Mol Cell Biol. 2001;21(16):5299–305.

71. Fukazawa T, Fujiwara T, Uno F, Teraishi F, Kadowaki Y, Itoshima T, Takata Y, Kagawa S, Roth JA, Tschopp J, Tanaka N. Accelerated degradation of cellular FLIP protein through the ubiquitin-proteasome pathway in p53-mediated apoptosis of human cancer cells. Oncogene. 2001;20(37):5225.

72. Ricci MS, Jin Z, Dews M, Yu D, Thomas-Tikhonenko A, Dicker DT, El-Deiry WS. Direct Repression of FLIP Expression by c-myc Is a Major Determinant of TRAIL Sensitivity. Mol Cell Biol. 2004;24(19):8541–55.

73. Pickart CM, Eddins MJ. Ubiquitin: structures, functions, mechanisms. Biochim Biophys Acta BBA - Mol Cell Res. 2004 Nov;1695(1–3):55–72.

74. Yang BF, Xiao C, Roa WH, Krammer PH, Hao C. Calcium/Calmodulin-dependent Protein Kinase II Regulation of c-FLIP Expression and Phosphorylation in Modulation of Fas-mediated Signaling in Malignant Glioma Cells. J Biol Chem. 2003;278(9):7043–50.

75. Zhang J, Chen Y, Huang Q, Cheng W, Kang Y, Shu L, Yin W, Hua ZC. Nuclear localization of c-FLIP-L and its regulation of AP-1 activity. Int J Biochem Cell Biol. 2009;41(8–9):1678–84.

76. Safa AR. c-FLIP, a master anti-apoptotic regulator. Exp Oncol. 2012;34(3):176.

77. Bartke T, Siegmund D, Peters N, Reichwein M, Henkler F, Scheurich P, Wajant H. p53 upregulates cFLIP, inhibits transcription of NF-[kappa] B-regulated genes and induces caspase-8-independent cell death in DLD-1 cells. Oncogene. 2001;20(5):571.

78. Park S-J, Sohn H-Y, Yoon J, Park SI. Down-regulation of FoxO-dependent c-FLIP expression mediates TRAIL-induced apoptosis in activated hepatic stellate cells. Cell Signal. 2009;21(10):1495–503.

79. Poukkula M, Kaunisto A, Hietakangas V, Denessiouk K, Katajamaki T, Johnson MS, Sistonen L, Eriksson JE. Rapid Turnover of c-FLIPshort Is Determined by Its Unique C-terminal Tail. J Biol Chem. 2005;280(29):27345–55.

80. Hershko A, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J Biol Chem. 1983 Jul 10;258(13):8206–14.

81. Özkaynak E, Finley D, Varshavsky A. The yeast ubiquitin gene: head-to-tail repeats encoding a polyubiquitin precursor protein. Nature. 1984 Dec;312(5995):663–6.

82. Chang L, Kamata H, Solinas G, Luo J-L, Maeda S, Venuprasad K, Liu YC, Karin M. The E3 Ubiquitin Ligase Itch Couples JNK Activation to TNFα-induced Cell Death by Inducing c-FLIPL Turnover. Cell. 2006;124(3):601–13.

83. Zhao L, Yue P, Khuri FR, Sun S-Y. mTOR Complex 2 Is Involved in Regulation of Cbl-Dependent c-FLIP Degradation and Sensitivity of TRAIL-Induced Apoptosis. Cancer Res. 2013;73(6):1946–57.

84. Burnett G, Kennedy EP. The enzymatic phosphorylation of proteins. J Biol Chem. 1954 Dec;211(2):969–80.

85. Cohen P. The regulation of protein function by multisite phosphorylation–a 25 year update. Trends Biochem Sci. 2000;25(12):596–601.

86. Cohen P. The structure and regulation of protein phosphatases. Annu Rev Biochem. 1989;58(1):453–508.

87. Kaunisto A, Kochin V, Asaoka T, Mikhailov A, Poukkula M, Meinander A,Eriksson JE. PKC-mediated phosphorylation regulates c-FLIP ubiquitylation and stability. Cell Death Differ. 2009;16(9):1215–26.

88. Shi B, Tran T, Sobkoviak R, Pope RM. Activation-induced Degradation of FLIPL Is Mediated via the Phosphatidylinositol 3-Kinase/Akt Signaling Pathway in Macrophages. J Biol Chem. 2009;284(21):14513–23.

89. Katayama R, Ishioka T, Takada S, Takada R, Fujita N, Tsuruo T, Naito M. Modulation of Wnt signaling by the nuclear localization of cellular FLIP-L. J Cell Sci. 2010;123(1):23–8.

90. Kataoka T, Tschopp J. N-Terminal Fragment of c-FLIP(L) Processed by Caspase 8 Specifically Interacts with TRAF2 and Induces Activation of the NF- B Signaling Pathway. Mol Cell Biol. 2004;24(7):2627–36.

91. Scaffidi C, Schmitz I, Zha J, Korsmeyer SJ, Krammer PH, Peter ME. Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. J Biol Chem. 1999;274(32):22532–22538.

92. Golks A, Brenner D, Krammer PH, Lavrik IN. The c-FLIP–NH 2 terminus (p22-FLIP) induces NF-κB activation. J Exp Med. 2006;203(5):1295–305.

93. Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW, Briand C, Grütter MG. The Long Form of FLIP Is an Activator of Caspase-8 at the Fas Death-inducing Signaling Complex. J Biol Chem. 2002;277(47):45162–71.

94. Chang DW, Xing Z, Capacio VL, Peter ME, Yang X. Interdimer processing mechanism of procaspase-8 activation. EMBO J. 2003;22(16):4132–4142.

95. Boatright KM, Cristina D, Denault J-B, Sutherlin DP, Salvesen GS. Activation of caspases-8 and-10 by FLIPL. Biochem J. 2004;382(2):651–657.

96. Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C,Hakem R, Salvesen GS, Green DR. Catalytic activity of the caspase-8–FLIPL complex inhibits RIPK3-dependent necrosis. Nature. 2011;471(7338):363–7.

97. Jun JI, Chung CW, Lee HJ, Pyo JO, Lee KN, Kim NS,Yoo HS, Lee TH, Kim E, Jung YK. Role of FLASH in caspase-8-mediated activation of NF-κB: dominant-negative function of FLASH mutant in NF-κB signaling pathway. Oncogene. 2005;24(4):688–96.

98. Barbero S, Mielgo A, Torres V, Teitz T, Shields DJ, Mikolon D, Bogyo M, Barilà D, Lahti JM, Schlaepfer D, Stupack DG. Caspase-8 Association with the Focal Adhesion Complex Promotes Tumor Cell Migration and Metastasis. Cancer Res. 2009;69(9):3755–63.

99. Oeckinghaus A, Ghosh S. The NF-?B Family of Transcription Factors and Its Regulation. Cold Spring Harb Perspect Biol. 2009 Oct 1;1(4):a000034–a000034.

100. Joyce D, Albanese C, Steer J, Fu M, Bouzahzah B, Pestell RG. NF-kappaB and cell-cycle regulation: the cyclin connection. Cytokine Growth Factor Rev. 2001;12(1):73–90.

101. Dohrman A, Kataoka T, Cuenin S, Russell JQ, Tschopp J, Budd RC. Cellular FLIP (Long Form) Regulates CD8+ T Cell Activation through Caspase-8-Dependent NF- B Activation. J Immunol. 2005;174(9):5270–8.

102. Neumann L, Pforr C, Beaudouin J, Pappa A, Fricker N, Krammer PH, Lavrik IN, Eils R. Dynamics within the CD95 death-inducing signaling complex decide life and death of cells. Mol Syst Biol. 2010;6:352.

103. Kataoka T, Budd RC, Holler N, Thome M, Martinon F, Irmler M, Burns K, Hahne M, Kennedy N, Kovacsovics M, Tschopp J. The caspase-8 inhibitor FLIP promotes activation of NF-κB and Erk signaling pathways. Curr Biol. 2000;10(11):640–648.

104. Kreuz S, Siegmund D, Rumpf J-J, Samel D, Leverkus M, Janssen O, Häcker G, Dittrich-Breiholz O, Kracht M, Scheurich P, Wajant H. NFκB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. J Cell Biol. 2004;166(3):369–80.

105. Hu W-H, Johnson H, Shu HB. Activation of NF-κB by FADD, Casper, and caspase-8. J Biol Chem. 2000;275(15):10838–10844.

106. Quintavalle C, Incoronato M, Puca L, Acunzo M, Zanca C, Romano G, Garofalo M, Iaboni M, Croce CM, Condorelli G. c-FLIPL enhances anti-apoptotic Akt functions by modulation of Gsk3β activity. Cell Death Differ. 2010 Dec;17(12):1908–16.

107. Gilot D, Serandour A-L, Ilyin GP, Lagadic-Gossmann D, Loyer P, Corlu A, Coutant A, Baffet G, Peter ME, Fardel O, Guguen-Guillouzo C. A role for caspase-8 and c-FLIPL in proliferation and cell-cycle progression of primary hepatocytes. Carcinogenesis. 2005 Dec 1;26(12):2086–94.

108. Motokura T, Arnold A. Cyclin D and oncogenesis. Curr Opin Genet Dev. 1993;3(1):5–10.

109. Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C, Strauss M. NF-κB Function in Growth Control: Regulation of Cyclin D1 Expression and G_0/G_1-to-S-Phase Transition. Mol Cell Biol. 1999 Apr 1;19(4):2690–8.

110. Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS. NF-κB Controls Cell Growth and Differentiation through Transcriptional Regulation of Cyclin D1. Mol Cell Biol. 1999 Aug 1;19(8):5785–99.

111. Wilkerson MJ. Principles and Applications of Flow Cytometry and Cell Sorting in Companion Animal Medicine. Vet Clin North Am Small Anim Pract. 2012 Jan;42(1):53–71.

112. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. J Immunol Methods. 1994 May 2;171(1):131–7.

113. Shapiro HM. Practical Flow Cytometry. John Wiley & Sons; 2005. 733 p.

114. Banks HT, Sutton KL, Thompson WC, Bocharov G, Roose D, Schenkel T, Meyerhans A. Estimation of Cell Proliferation Dynamics Using CFSE Data. Bull Math Biol. 2011 Jan;73(1):116–50.

115. Hasenauer J, Schittler D, Allgöwer F. Analysis and Simulation of Division- and Label-Structured Population Models: A New Tool to Analyze Proliferation Assays. Bull Math Biol. 2012 Nov;74(11):2692-732

116. Gratzner HG. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science. 1982 Oct 29;218(4571):474–5.

117. Buck S, Bradford J, Gee K, Agnew B, Clarke S, Salic A. Detection of S-phase cell cycle progression using 5-ethynyl-2′-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2′-deoxyuridine antibodies. BioTechniques. 2008 Jun;44(7):927–9.

118. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes. Angew Chem Int Ed. 2002 Jul 15;41(14):2596–9.

119. Krishan A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol. 1975;66(1):188–193.

120. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods. 1991 Jun 3;139(2):271–9.

121. Doncic A, Falleur-Fettig M, Skotheim JM. Distinct interactions select and maintain a specific cell fate. Mol Cell. 2011 Aug 19;43(4):528–39.

122. Haass NK, Beaumont KA, Hill DS, Anfosso A, Mrass P, Munoz MA, Kinjyo I, Weninger W. Real-time cell cycle imaging during melanoma growth, invasion, and drug response. Pigment Cell Melanoma Res. 2014 Sep;27(5):764–76.

123. Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H, Imamura T, Ogawa M, Masai H, Miyawaki A. Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression. Cell. 2008 Feb 8;132(3):487–98.

124. Gavvovidis I, Rost I, Trimborn M, Kaiser FJ, Purps J, Wiek C, Hanenberg H, Neitzel H, Schindler D. A Novel MCPH1 Isoform Complements the Defective Chromosome Condensation of Human MCPH1-Deficient Cells. Fugmann SD, editor. PLoS ONE. 2012 Aug 30;7(8):e40387.

125. Kaltschmidt B, Kaltschmidt C, Hehner SP, Droge W, Schmitz ML. Repression of NF-kB impairs HeLa cell proliferation by functional interference with cell cycle checkpoint regulators. Oncogene. 1999;18(21):3213–3225.

126. Micheau O. Cellular FLICE-inhibitory protein: an attractive therapeutic target? Expert Opin Ther Targets. 2003 Aug;7(4):559–73.

127. Siegmund D, Hadwiger P, Pfizenmaier K, Vornlocher H-P, Wajant H. Selective inhibition of FLICE-like inhibitory protein expression with small interfering RNA oligonucleotides is sufficient to sensitize tumor cells for TRAIL-induced apoptosis. Mol Med Camb Mass. 2002 Nov;8(11):725–32.

128. Stagni V, Mingardi M, Santini S, Giaccari D, Barilà D. ATM kinase activity modulates cFLIP protein levels: potential interplay between DNA damage signalling and TRAIL-induced apoptosis. Carcinogenesis. 2010 Nov;31(11):1956–63.

129. Galligan L, Longley DB, McEwan M, Wilson TR, McLaughlin K, Johnston PG. Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. Mol Cancer Ther. 2005 Dec;4(12):2026–36.

130. Mawji IA, Simpson CD, Gronda M, Williams MA, Hurren R, Henderson CJ, Datti A, Wrana JL, Schimmer AD. A chemical screen identifies anisomycin as an anoikis sensitizer that functions by decreasing FLIP protein synthesis. Cancer Res. 2007 Sep 1;67(17):8307–15.

131. Park SJ, Kim MJ, Kim HB, Sohn HY, Bae JH, Kang CD, Kim SH. Trichostatin A sensitizes human ovarian cancer cells to TRAIL-induced apoptosis by down-regulation of c-FLIPL via inhibition of EGFR pathway. Biochem Pharmacol. 2009 Apr 15;77(8):1328–36.